**Certificate of Verification**

I, William C. Rice, residing at 2 Steeple Chase Court, Durham, NC, USA, hereby state that I am well acquainted with the German and English languages and that, to the best of my knowledge, the attached documents are an accurate translation of the Offenlegungsschrift (Published Application) **DE10 2004 011 503 A1** 2005.09.15 and the Patent Application "Verfahren zur Identifizierung und Quantifizierung von tumor-assoziierten Peptiden" **DE10 2004 005 273.5** into the English language.

Durham, North Carolina February 23, 2010

Place, Date

William C. Rice

signature

**Attached Drawings**

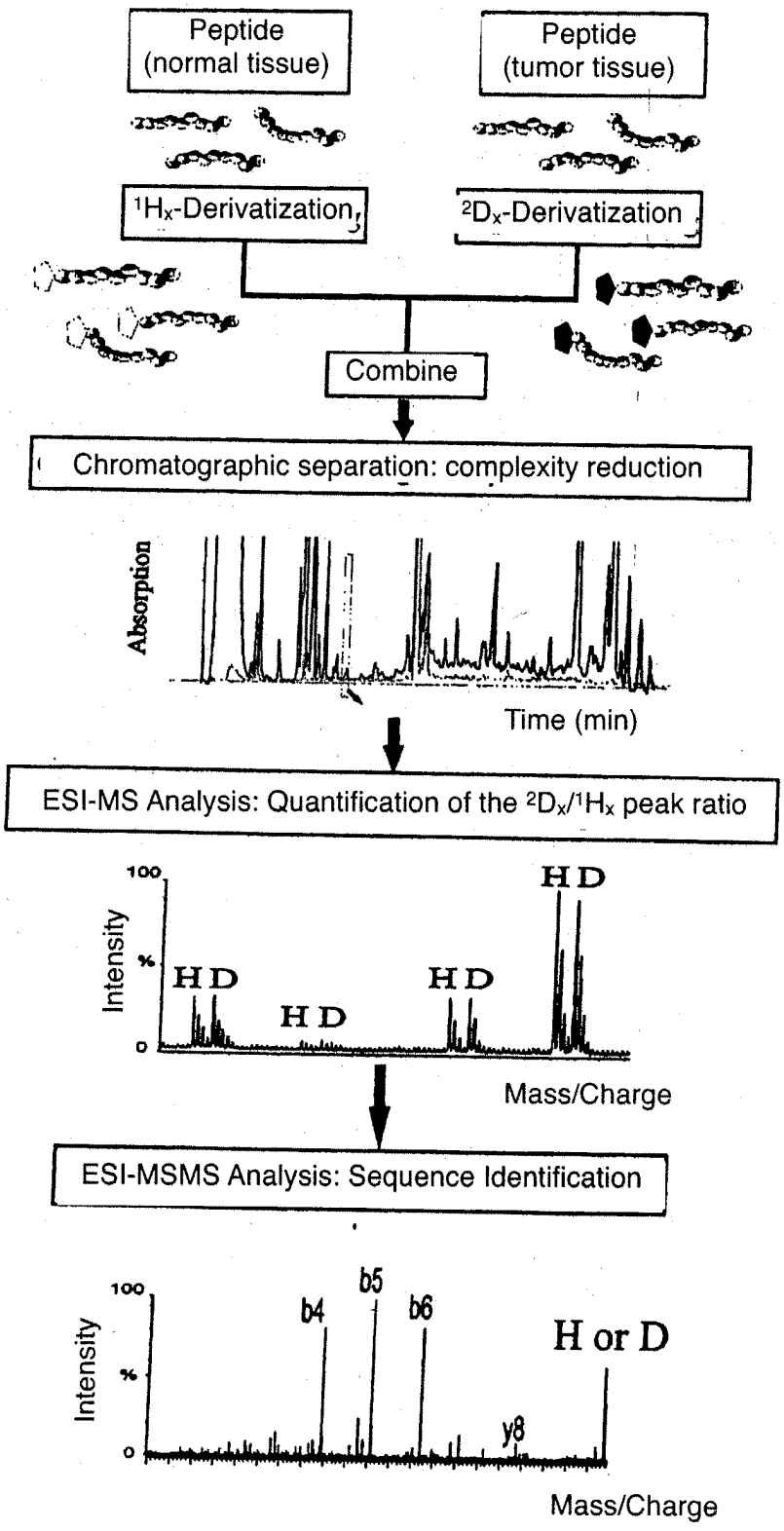
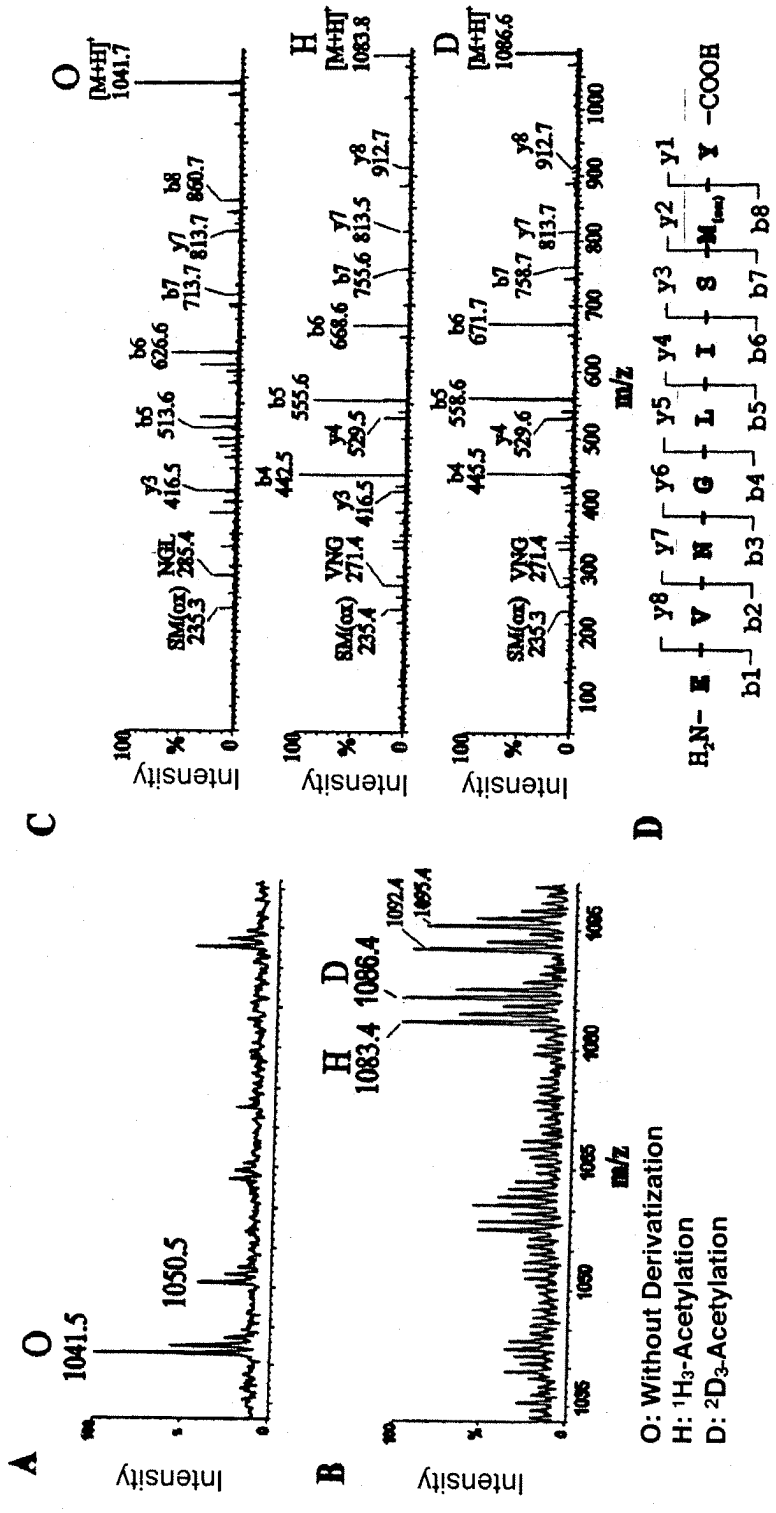



**Fig. 1**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Intensity
B
H
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D
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1092.4
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m/z
C
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[M+H]+
1041.7
H
[M+H]+
1083.8
D
[M+H]+
1086.6
Intensity
SM4(ox)
235.3
NGL
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y3
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b6
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b7
713.7
y7
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b8
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b4
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y4
529.5
b5
555.6
b6
668.6
b7
755.6
y8
912.7
b4
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b5
558.6
b6
671.7
b7
758.7
m/z
D
H2N-
V
N
G
L
I
S
Y
-COOH
y8
y7
y6
y5
y4
y3
y2
y1
b1
b2
b3
b4
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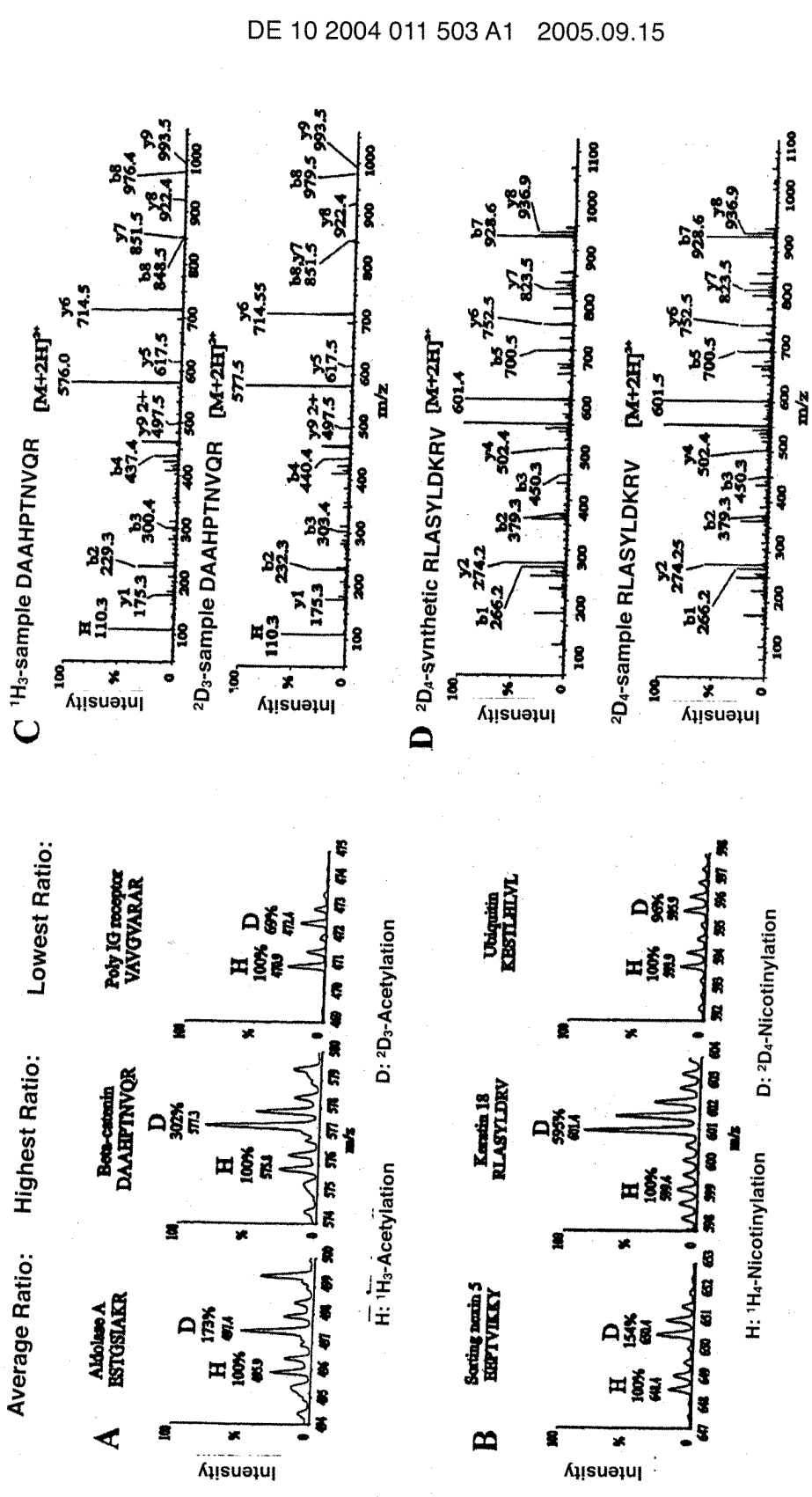
b6
b7
b8
O: Without Derivatization
H: 1H3-Acetylation
D: 2D3-Acetylation

Fig. 2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Highest Ratio:
Lowest Ratio:
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Beta-catenin
DAAHPTNVQR
Poly IG receptor
VAVGVARAR
H: $^{1}H_3$-Acetylation
D: $^{2}D_3$-Acetylation
B
Sorting nexin 5
HKPTVIKKY
Keratin 18
RLASYLDKV
Ubiquitin
KESTLHLVL
H: $^{1}H_4$-Nicotinylation
D: $^{2}D_4$-Nicotinylation
C
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D
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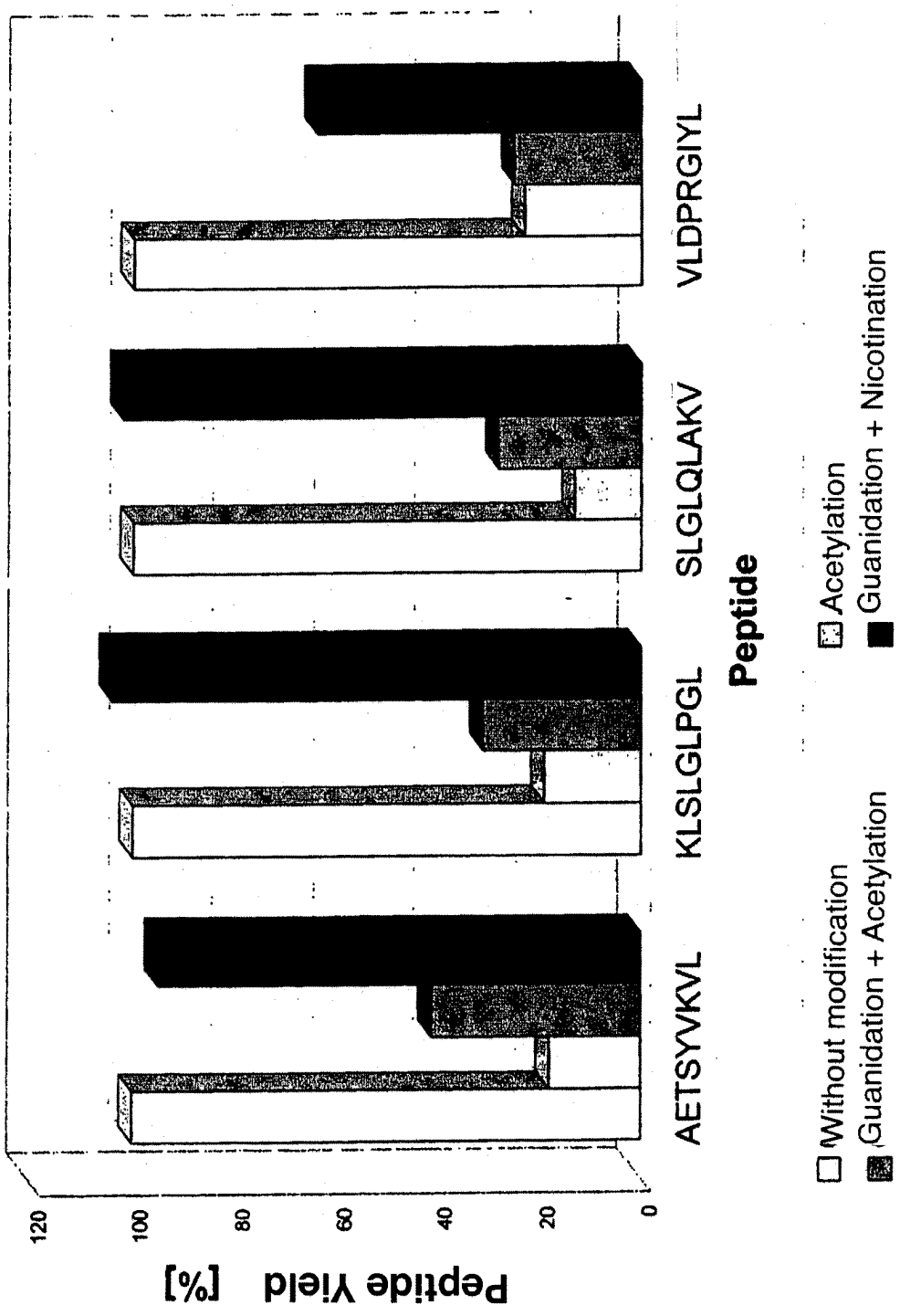
Intensity
m/z

Fig. 3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100
80
60
40
20
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KLSLGLPGL
SLGLQLAKV
VLDPRGIYL
Peptide
Without modification
Acetylation
Guanidation + Acetylation
Guanidation + Nicotination

Fig. 4

**Attached Drawings**

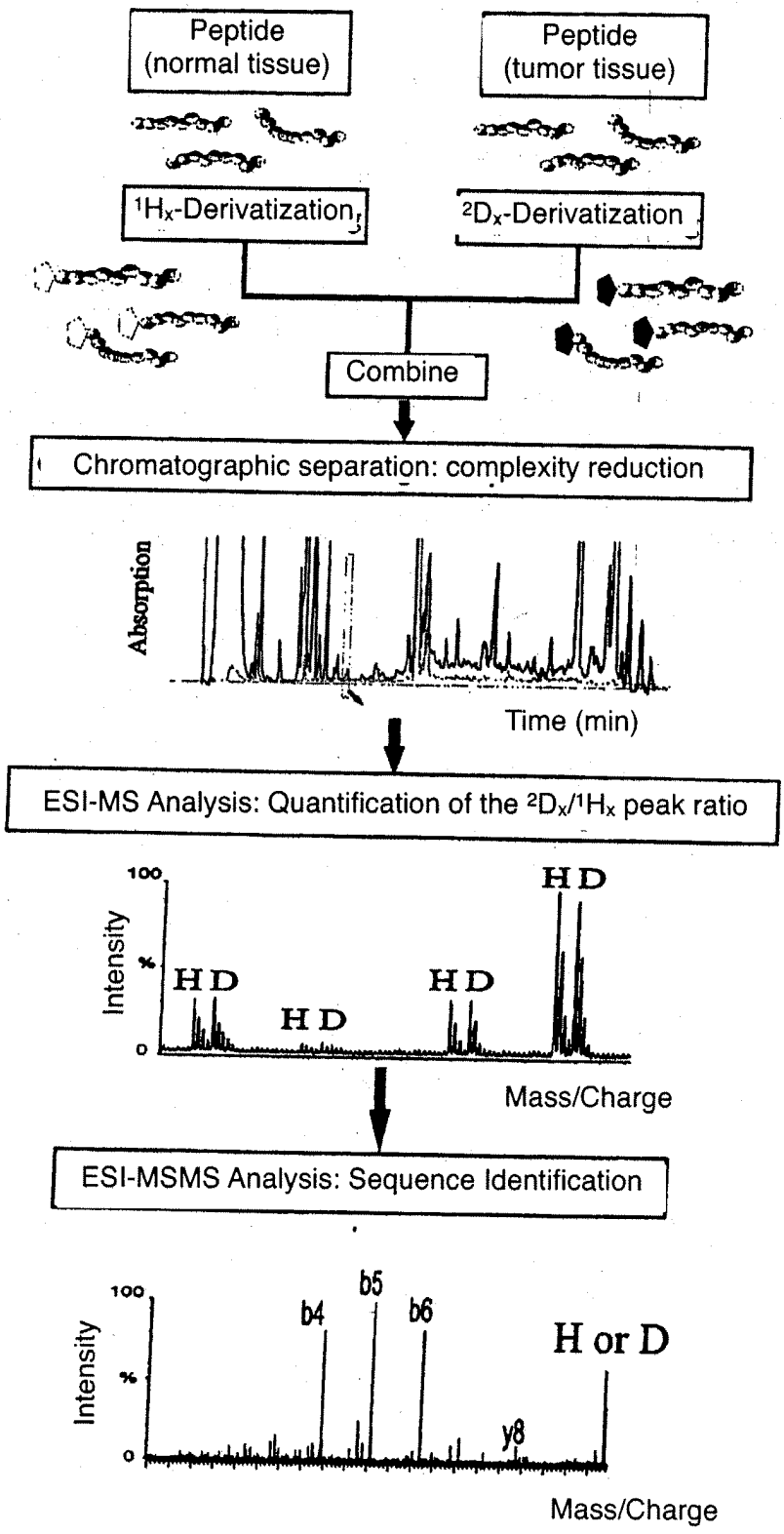
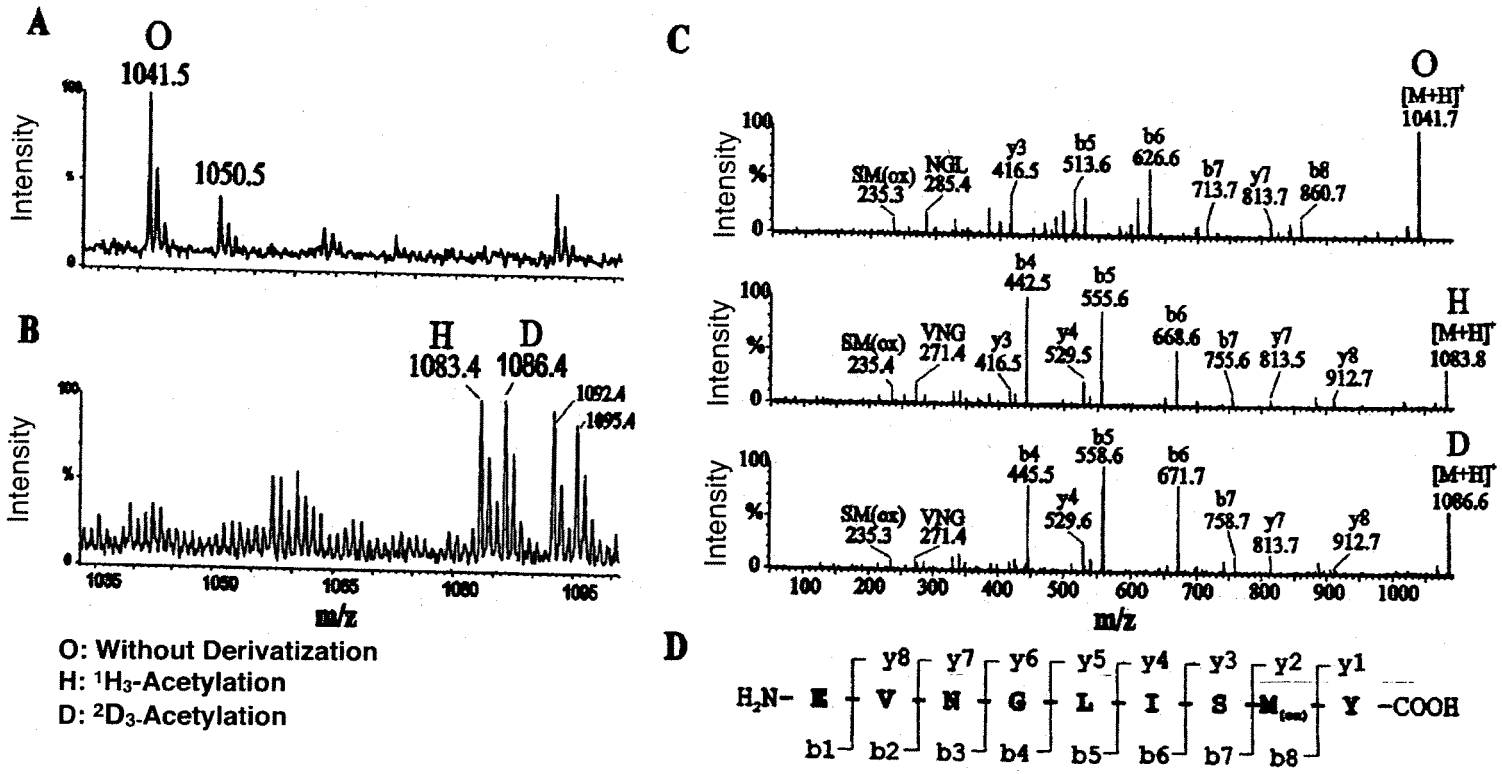



**Fig. 1**


A
O
1041.5
1050.5
Intensity
B
H
1083.4
D
1086.4
1092.4
1095.4
Intensity
m/z
O: Without Derivatization
H: $^{1}H_3$-Acetylation
D: $^{2}D_3$-Acetylation
C
O
[M+H]+
1041.7
Intensity
SIM(ox) 235.3
NGL 285.4
y3 416.5
b5 513.6
b6 626.6
b7 713.7
y7 813.7
b8 860.7
H
[M+H]+
1083.8
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b5 555.6
SIM(ox) 235.4
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y4 529.5
b6 668.6
b7 755.6
y7 813.5
y8 912.7
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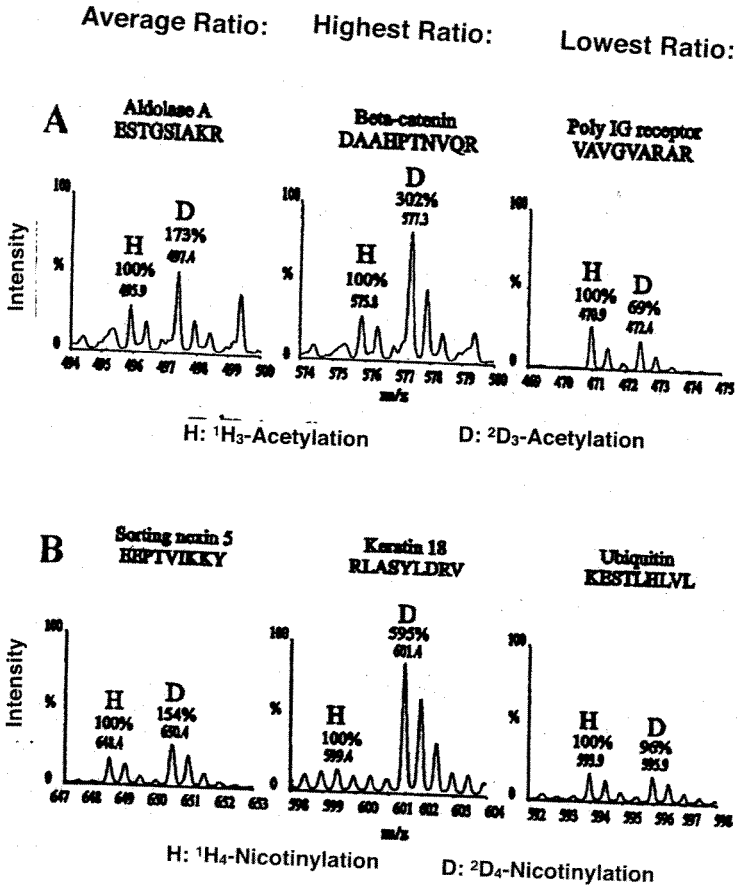
[M+H]+
1086.6
b4 445.5
b5 558.6
b6 671.7
SIM(ox) 235.3
VNG 271.4
y4 529.6
b7 758.7
y7 813.7
y8 912.7
100 200 300 400 500 600 700 800 900 1000
m/z
D
H2N- E V N G L I S M(ox) Y -COOH
y8 y7 y6 y5 y4 y3 y2 y1
b1 b2 b3 b4 b5 b6 b7 b8

Fig. 2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Highest Ratio:
Lowest Ratio:
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Aldolase A
ESTGSIAKR
Beta-catenin
DAAHPTNVQR
Poly IG receptor
VAVGVARAR
Intensity
H
100%
D
173%
H
100%
D
302%
H
100%
D
69%
m/z
H: 1H3-Acetylation
D: 2D3-Acetylation
B
Sorting nexin 5
HRPTVIKKY
Keratin 18
RLASYLDRV
Ubiquitin
KESTLHLVL
Intensity
H
100%
D
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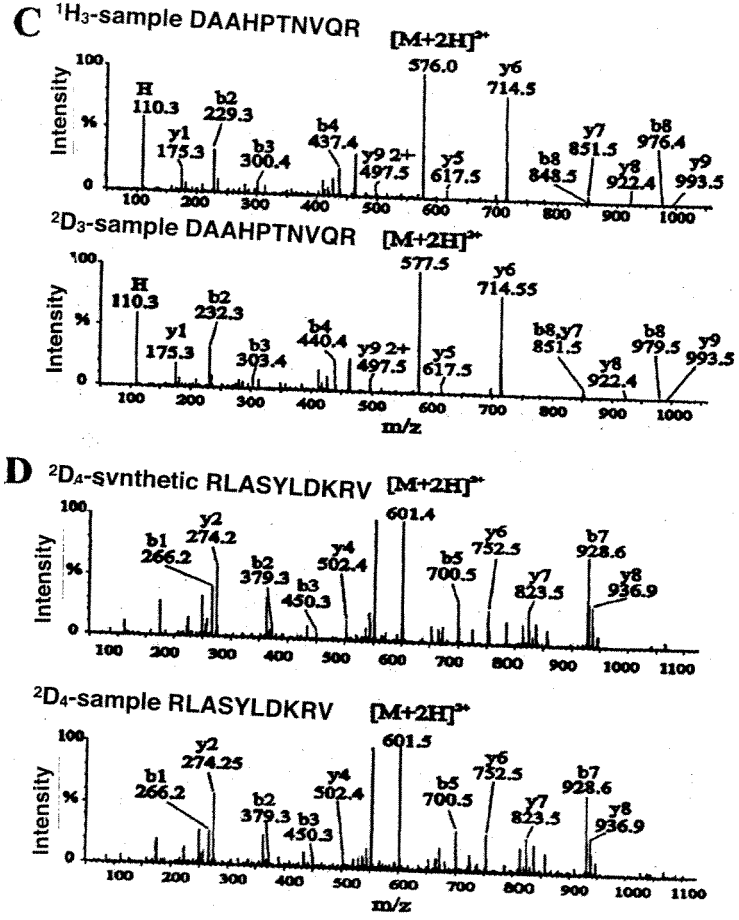
H: 1H4-Nicotinylation
D: 2D4-Nicotinylation


C
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[M+2H]2+
Intensity
H
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b2
229.3
y1
175.3
b3
300.4
b4
437.4
y9 2+
497.5
576.0
y6
714.5
y5
617.5
y7
851.5
b8
848.5
b8
976.4
y8
922.4
y9
993.5
2D3-sample DAAHPTNVQR
[M+2H]2+
H
110.3
b2
232.3
y1
175.3
b3
303.4
b4
440.4
y9 2+
497.5
577.5
y6
714.55
y5
617.5
b8,y7
851.5
y8
922.4
b8
979.5
y9
993.5
m/z


D
2D4-synthetic RLASYLDKRV
[M+2H]2+
Intensity
b1
266.2
y2
274.2
b2
379.3
b3
450.3
y4
502.4
601.4
b5
700.5
y6
752.5
y7
823.5
b7
928.6
y8
936.9
2D4-sample RLASYLDKRV
[M+2H]2+
b1
266.2
y2
274.25
b2
379.3
b3
450.3
y4
502.4
601.5
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823.5
b7
928.6
y8
936.9
m/z

Fig. 3

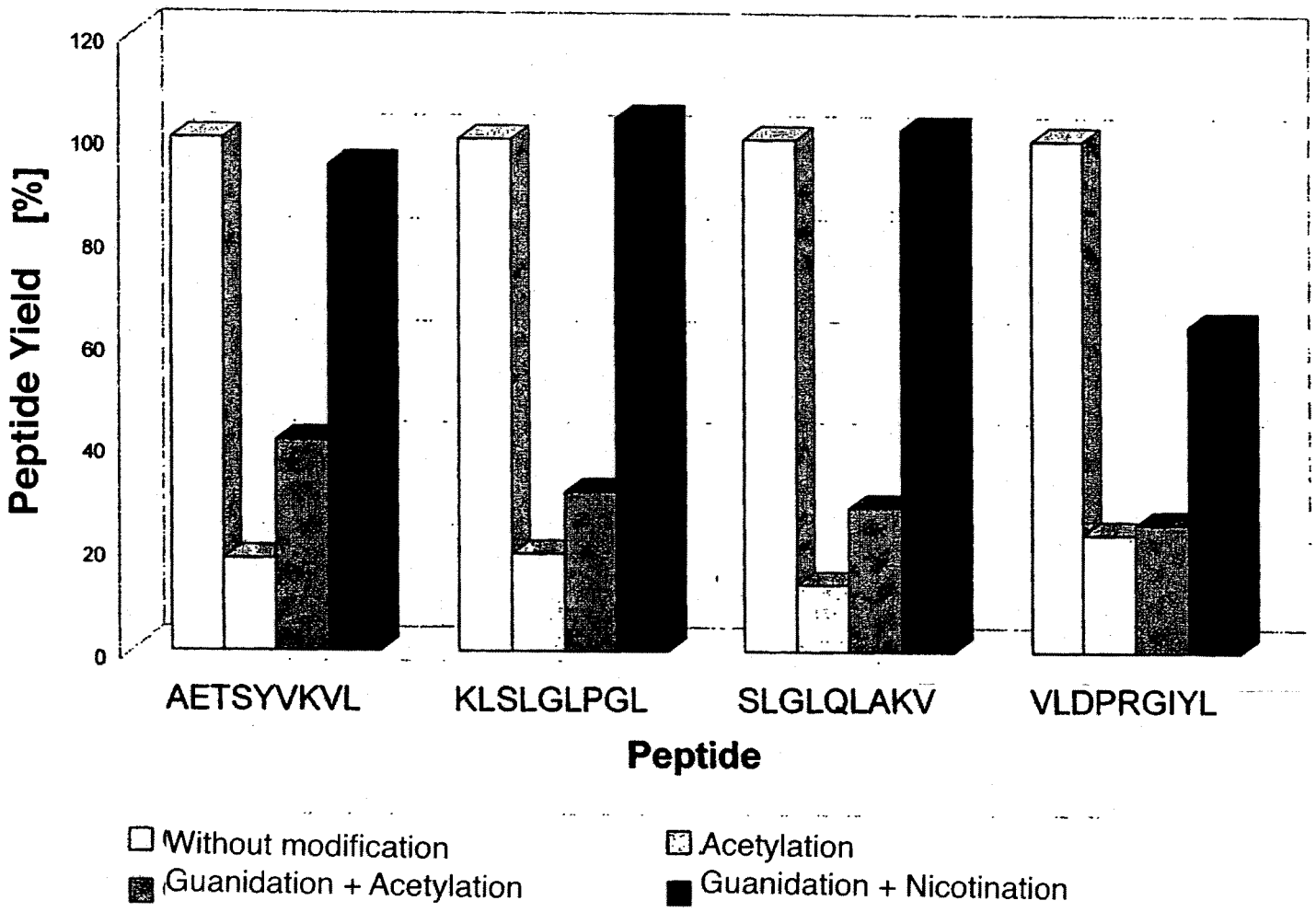
Peptide Yield [%]
120
100
80
60
40
20
0
AETSYVKVL
KLSLGLPGL
SLGLQLAKV
VLDPRGIYL
Peptide
Without modification
Acetylation
Guanidation + Acetylation
Guanidation + Nicotination

Fig. 4

# Method for identification and quantification of tumor-associated peptides

The invention relates to a method for identifying and quantifying tumor-associated peptides and to a method for preparing tumor-associated peptides and to the peptides identified/quantified/prepared thereby as well as their use.

Such peptides are, for example, used in the immunotherapy of tumor diseases.

The recognition of tumor-associated antigens by immune system components plays a major role in the elimination of tumor cells by the immune system. This mechanism is based upon the fact that there exist qualitative or quantitative differences between tumor cells and normal cells. In order to generate an immune system response directed at the tumor, the tumor cells must express antigens against which an immune response sufficient to eliminate the tumor can be induced.

CD8-expressing cytotoxic T-lymphocytes (hereinafter referred to as CTL) play a large role in the elimination of tumors. To trigger an immune reaction of this type by CTL, foreign proteins/peptides must be presented to the CTL. T cells recognize antigens as peptide fragments only if they are presented on cell surfaces by MHC ("major histocompatibility complex") molecules. These MHC molecules are peptide receptors that normally bind peptides within the cell in order to transport them to the cell surface. This peptide/MHC molecule complex can be recognized by T cells. Human MHC-molecules are also called human leukocyte antigens (HLA).

In the past, antigen-specific immunotherapy based on T-cells has proven successful in the treatment of cancer.

Induction of a specific CTL response directed against a tumor is dependent on identification of MHC class I-ligands derived from tumor-associated antigens (TAA). Such tumor-associated antigens can be exclusively present in malignant cells, for example as products of mutated genes. Other important classes of tumor-associated antigens are tissue-specific structures such as melanocyte-differentiating antigens. A third class of tumor-associated antigens are proteins that are overexpressed in tumors.

The methods for identification and characterization of TAA, which are the starting point for a therapeutic vaccine, are - on the one hand - based on the stimulation of CTL or antibodies that are already present in the patient. This immunological approach is combined either with an analysis of the gene expression profile or with a mass-spectrometry (MS) sequencing of the identified genes (see van der Bruggen, et al., 1991, A gene encoding an antigen recognized by cytolytic T-lymphocytes on a human melanoma, Science 254: 1643-1647, and Cox et al., 1994, Identification of a peptide recognized by five melanoma-specific human cytotoxic T-cell lines, Science 264:716-719). Methods for identifying TAA, which are based on the comparative analysis of the transcription profile of tumor tissue and normal tissue, are, for example, the use of traditional DNA chip technology and processes for the hybridization of messenger-RNA from the tissue samples to be compared.

Celis et al., 1994, Induction of anti-tumor cytotoxic T-lymphocytes in normal humans using primary cultures and synthetic peptide epitopes, Proc. Natl. Acad. Sci. USA 91: 2105-2109, applied a method which takes advantage of the prediction of MHC class I-ligands derived from a selected TAA, and in which these ligands were verified experimentally as T-cell epitopes in a next step.

It is disadvantageous in this procedural method, which necessitates the availability of T-cells from patients as a prerequisite, that the experimental use and cultivation is very expensive.

Schirle et al., 2000, Identification of tumor-associated MHC class I ligands by a novel T-cell independent approach, Eur. J. Immunol. 30:2216-2225, describe a method not dependent on T-cells in which the prediction of MHC class I ligands is combined with the intended search for the predicted peptide ligands in complex peptide mixtures, whereby the peptides were identified by the combination of highly sensitive capillary liquid chromatogrphy with mass spectroscopy (LC-MS).

See for example Young et al., 2001, Expression profiling of renal epithelial neoplasms: a method for tumor classification and discovery of molecular markers, Am. J. Pathol., 158:1639-1651, show that using analyses with DNA chip technology, a large portion of TAA from individual tumors can be identified. MHC class I ligands derived from overexpressed, selectively or exclusively expressed proteins provide potential targets for a CTL-based elimination of tumors. Mathiassen et al, 2001, Tumor-associated antigens identified by

mRNA expression profiling induce protective anti-tumor immunity, Eur. J. Immuno. 31: 1239-1246, demonstrated, in a mouse model, that by combining gene expression analysis with epitope prediction, a successful vaccine can be prepared.

The disadvantage of the epitope prediction is due to the fact that for a small number of TAA, a very large number of possible MHC class I ligands is specified, the majority of which is actually not presented by MHC class I molecules at all. This is why the majority of the only predicted epitopes can not induce a CTL-based elimination of tumors.

Weinschenk et al., 2002, Integrated functional genomics approach for the design of patient-individual antitumor vaccines, Cancer Res. 62:5818-5827, show that by the combination of a gene expression analysis with the MHC class I ligands of a tumor, which were isolated and analyzed by liquid chromatography and mass spectroscopy, intended candidates for the composition of a therapeutic vaccine can be determined in one method. The big advantage compared to the exclusive use of gene expression analysis or mass spectroscopy lies in the fact that the MHC class I ligands are analyzed from a complex peptide mixture and they are suitable to a certain extent as immunoreactive peptides due to the fact that they are not only actually presented by MHC class I molecules but also derived from genes which become expressed exclusively, selectively or especially highly in tumors.

The combined method from gene expression analysis and mass spectroscopy, however, has the disadvantage that the quantitative ratio, between tumor tissue and normal tissue, of the peptides actually being presented by MHC class I molecules is not determined. In order to induce a CTL response which is directed against MHC class I ligands of TAA presented high on the tumor and low on the normal tissue, the quantitative ratio of peptides presenting MHC class I ligands must be determined.

Thus it is the purpose of the present invention to provide a new method with which in a simple and intentional way, immunoreactive peptides can be identified and the quantitative ratios of these tumor-associated peptides can be determined comparatively for tumor and normal tissue.

The purpose is achieved according to the invention by a method of identifying and quantifying tumor-associated peptides in which at first at least two different sources (tumor

and normal tissue or correspondingly transfected cell lines), of the same quantity by weight or cell number, are prepared for extraction of the peptides, and the peptides from the different sources are then chemically modified in an identical manner separately from each other using at least two different stable isotopes of the same element, the peptides modified in this way are then mixed and after that preferably isolated by chromatographic methods and the amino acid sequences of the peptides are determined, so that the determination of the relative quantitative ratios of sequence-identical peptides from different samples to each other is done using the stable isotopes used in the chemical modification.

In order to ensure that the starting material from the two sources has the same quantity (weight in tissue) or cell number, a normalization can also be done using, for example, a peptide uniformly occuring in the tumor tissue and normal tissue, or another marker.

Furthermore, the invention involves a tumor-associated peptide with an amino acid sequence chosen from the group consisting of SEQ-ID no. 1 to 36 from the attached sequence protocol, whereby the peptide is capable of binding to a molecule of the human major histocompatibility complex (MHC) class I.

The invention involves, furthermore, the use of peptides for manufacturing a drug and for treating tumor diseases and/or adenomatous diseases.

A method according to the invention for identifying and quantifying tumor-associated peptides thus includes the following steps:

a) Preparing a sample from tumorous tissue and a sample from corresponding normal tissue or correspondingly transfected cell lines, whereby both samples have the same quantities by weight or cell numbers,

b) Isolation of peptides from the sample made from tumorous tissue,

c) Isolation of peptides from the sample made from the corresponding normal tissue,

d) Chemical change of the peptides obtained from step (b) with a chemical group that

contains a stable isotope of an element from the Periodic Table of the Elements (e.g. Deuterium, $^{2}D$),

e) Chemical change of the peptides obtained from step (c) with a chemical group that contains a second stable isotope of an element used in step d) from the Periodic Table of the Elements (e.g. Hydrogen, $^{1}H$),

f) Mixing of the chemically modified peptides obtained from steps (d) and (e),

g) Separation of the peptides obtained from step f) by a chromatographic process,

h) Identification and determination of peptides having identical amino acid sequences and the quantitative ratios of chemically modified peptides with identical amino acid sequences from step (g),

i) Identification of tumor-associated peptides with applicability, preferably excellent applicability for the composition of a therapeutic vaccine based on the data obtained from step (h).

The inventors have recognized that by the method based preferably on mass spectroscopy and on differential chemical modification for determining the differences in the quantitative ratios of peptides between tumor tissue and normal tissue, peptides can be identified that are especially suitable for the composition of therapeutic vaccines.

With the method according to the invention it is thus possible to identify peptides which are suitable for the individual composition, for example, of a personalized mixture of tumor-associated peptides for an individual patient, whereby the peptides can then induce a targeted CTL response adapted to the individual patient's requirement.

For example, industrial laboratories - after having received patient samples - can systematically and efficiently perform this method, and can - after having identified suitable peptides - provide clinics in charge with the peptide sequences; the clinics can then synthesize and formulate the peptides as a therapeutic vaccine. However, it is also possible that a laboratory is carrying out identification as well as preparation, formulation, and

production of the tumor-associated peptides suitable for the respective patient.

Also, the systematic and frequent application of the method can lead to a commercial use of suitable tumor-associated peptides, which are found especially frequently as MHC class I ligands, as finished drugs.

The new method is thus applicable both in the context of a pure service as well as in connection with production, formulation and preparation, both for an individual patient, as well as on a suitable industrial scale for use by businesses of the pharmaceutical industry.

In a preferred embodiment, the peptides isolated in steps (b) and (c) are MHC class I ligands.

Only peptides which are bound to MHC-molecules can induce a CTL immune response. Peptides which are derived, for example, from overexpressed genes of a tumor but which are not bound to MHC-molecules, do not induce a CTL immune reaction. Thus, not all peptides, e.g. those peptides only determined by prediction of epitopes, are actually suitable for inducing an immune response.

In an additional preferred embodiment form, step (d) is performed via the guadinylation of the ε-amino group of a lysine residue of a peptide by chemical reaction of peptides with O-methylisourea hemisulfate and the nicotinylation of the α-amino group by chemical reaction of peptides with $^{2}D_{4}$-nicotinyl amino hydroxy succinimide ($^{2}D_{4}$-NicNHS). The guadinylation of the ε-amino group of the lysine residues of peptides is decribed, for example, in Beardsley et al., 2002, Optimization of guadination procedures for MALDI mass mapping, Anal. Chem. 74:1884-1890. The nicotinylation of the a-amino group of peptides is described, for example, in Munchbach et al., 2000, Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation-directing moiety, Anal. Chem. 72:4047-4057.

In an additional preferred embodiment form, step (e) is performed via the guadinylation of the ε-amino group of a lysine residue of a peptide by chemical reaction of peptides with O-methylisourea hemisulfate and the nicotinylation of the α-amino group by chemical reaction of peptides with $^{1}H_{4}$-nicotinyl amino hydroxy succinimide ($^{1}H_{4}$-NicNHS).

In an additional preferred embodiment form, the analysis in steps (g) and (h) is done using a coupled liquid chromatography and mass spectroscopy process. Using this technique, the individual chemically modified peptides can be determined exactly and efficiently and with a high throughput. The use of mass spectroscopy to determine chemically modified peptides is, for example, described in Munchbach et al., 2000, Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation directing moiety, Anal. Chem. 72:4047-4057. The identification of peptides from tumor tissue is described, for example, in Weinschenk et al., 2002, Integrated functional genomics approach for the design of patient-individual antitumor vaccines, CancerRes. 62:5818-5827.

In an additional preferred embodiment form, an additional step is performed according to step (h) in which the reactivity of leukocytes from the peripheral blood, preferably T-lymphocytes, against the peptides defined by step (h), is tested.

A further object is the method according to the invention, wherein the reactivity of peripheral leukocytes against the peptides defined by step (h) is tested by means of measuring γ-Interferon-mRNA and/or cytokin-mRNA synthesized by the leukocytes.

By detecting γ-Interferon-mRNA and/or cytokin-mRNA, it is possible to precisely prove the specific reactivity of leukocytes, preferably of T-lymphocytes against antigenic peptides. Both substances are secreted by activated T-lymphocytes after their activation by corresponding peptides which are bound to MHC-molecules on the cell surfaces. With this additional step candidates of the already identified peptides can be identified even more precisely.

Yet another object is the method according to the invention, wherein, following step (h), a further step is performed, in which the presence of the specific T-lymphocytes is detected.

Using this method it is possible to specifically detect to what extent T-lymphocytes directed against isolated and identified peptides are pre-existing in patients. By performing this step it is possible to apply, as a vaccine, only those peptides for which T-lymphocytes are already pre-existing in the patient. The peptides can then be used to activate these specific T-lymphocytes.

A further object is the method according to the invention, wherein the detection of specific pre-existing T-lymphocytes is performed by labeling the leukocytes with reconstituted complexes of MHC molecules and antigenic peptide.

With this method the so-called tetramer-technology is utilized. A method for generating such reconstituted complexes ("tetramers") and for utilizing them is disclosed, for example, in Altman et al., 1996, Phenotypic analysis of antigen-specific T-lymphocytes, Science 274:94-96.

In an additional preferred method, specific T-lymphocytes from the peripheral blood of patients with reconstituted complexes made of MHC molecules and antigenic peptides, which are bound together with the molecule CD28 to a synthetic surface, are activated. This method is described, for example, in Walter et al., 2003, Cutting Edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres, J. Immunol. 171:4974-4979.

The invention involves, in an additional aspect, immunoreactive peptides which are identified and/or produced by the method according to the invention.

These peptides can be produced after identification in an intentional and specific manner.

Another object of the invention is a pharmaceutical composition comprising one or more peptides which have been identified and/or prepared by the method according to the invention.

The composition may be applied, for example, parenterally, for example subcutaneously, intradermally or intramuscularly. In the process, the peptides are dissolved or suspended in a pharmaceutical carrier; furthermore, the composition can contain excipients, such as for example, buffers, binders, etc. A comprehensive description of excipients, as they can be used in this type of composition, for example, is described in A. Kibbe, 2000, Handbook of Pharmaceutical Excipients, 3. Ed., American Pharmaceutical Association and Pharmaceutical Press. The peptides can also be administered together with immunostimulating substances, such as, for example, cytokines. A comprehensive description of immunostimulating substances, as they can be administered together with peptides, is given, for example, in

Ribas et al., 2003, Current developments in cancer vaccines and cellular immunotherapy, J. Clin. Oncol. 21:2415-2432.

According one object of the invention the peptide may be used for treatment of tumor diseases and for preparing a medicament for treatment of tumor diseases.

Tumor diseases to be treated comprise renal, lung, intestinal, gastric, pancreas, breast, prostate, ovarial and/or skin cancer. This list of tumor diseases is only exemplary, and is not intended to limit the area of application.

The peptides can further be used for assessment of the therapy-course of a tumor disease.

Also, for other vaccines or therapies, peptides can be used to evaluate a course of treatment. Thus, the peptides according to the invention may not only be used for therapeutic, but also for diagnostic purposes.

In an additional embodiment form of the invention, peptides are used to produce antibodies.

Polyclonal antibodies may be obtained, in a conventional way, by the immunization of animals by means of injection of the peptides and the subsequent purification of the immunoglobulins from the blood of the immunized animals.

Monoclonal antibodies can be produced by standard protocols, such as described, for example, in Methods Enzymol., 1986, Hybridoma technology and monoclonal antibodies, 121:1-947.

Bispecific monoclonal antibodies can be produced according to standard protocols, such as, for example, Tomlinson et al., 2000, Methods for generating multivalent and bispecific antibody fragments, Methods Enzymol. 346:461-479.

In another aspect, the invention involves nucleic acid molecules which code for the peptide isolated by the method according to the invention.

The nucleic acid molecules can be DNA- or RNA-molecules and can be used for immune therapy of cancer as well.

According to one object of the invention the nucleic acid molecules can be provided in a vector.

A further object of the invention is a cell genetically modified by means of the nucleic acid molecule such that the cell produces a peptide identified according to the invention.

Another object of the invention is a method for preparing a tumor-associated peptide with which a peptide is identified according to the disclosed method and the identified peptide is synthesized chemically, *in vitro* or *in vivo.*

Peptides can be prepared by chemical reaction of amino acids, for example by the method of Merrifield, which is known in the art (see Merrifield, 1963, J. Am. Chem. Soc. 85:2149-2154).

Peptides can be produced in vitro, for example, in cell-free expression systems. Peptides can be manufactured in vivo in prokaryotic and eukaryotic cells.

A preferred embodiment form of the present invention is a method for producing a vaccine with the steps

(a) performing the disclosed method,

(b) producing the tumor-associated peptides identified in step (i)

(c) formulating the tumor-associated peptides produced in step (j)

It is understood that aforementioned and subsequently to be explained characteristics can be applied not only in the respective combination given, but also can be used in other combinations or alone, without leaving the context of the invention presented here.

Embodiments of the invention are displayed and explained in the figures and the example below. Description of the drawings

**Fig. 1** an overview of the method according to the invention for identification and quantification of tumor-associated peptides;

**Fig. 2** the mass spectroscopic analysis of (A) non-modified and (B) $^{1}H_3/^{2}D_3$-acetylated peptides. ( C) Mass spectroscopic analysis of a peptide mixture which, for example, contains both the non-modified peptide, the $^{1}H_3$-acetylated peptide and $^{2}D_3$-acetylated peptides with the amino acid sequence EVNGLISMY; (D) expains the nomenclature used in **Fig. 2**;

**Fig. 3** a comparative quantification of antigenic peptides from two different sources, where in (A) a mass-spectroscopic analysis is shown of the relative quantitative ratios of three different peptides made from two tissue samples (intestinal cancer sample, sample of normal tissue from the same patient). The peptides isolated from the intestinal cancer sample were $^{2}D_3$-acetylated. The peptides isolated from the normal tissue sample were $^{1}H_3$-acetylated. (B ) shows a mass-spectroscopic analysis of three different peptides made of $^{1}H_4$-nicotinylated/guadinylated Awells cells and with keratin 18-transfected and $^{2}D_4$- nicotinylated / guadinylated Awells cells. (C) shows the determination of amino acid sequences of a $^{1}H_3$-acetylated peptide with the amino acid sequence DAAHPTNVQR and a $^{2}D_3$-acetylated peptide with the amino acid sequence DAAHPTNVQR by fragmentation;

**Fig. 4** Yields of peptides chemically modified in different ways. Four peptides with the amino acid sequences AETSYVKVL, KLSLGLPGL, SLGLQLAKV and VLDPRGIYL were added into a mixture in equimolar portions and then for the purpose of a comparative study of three procedural methods for chemical modification, they were either acetylated, or acetylated and guanidinylated, or guanidinylated and nicotinylated. After the conclusion of the chemical reaction for modification of the reference peptides, they were mixed with the non-modified peptides used at the beginning in order to then make a comparison possible in an analytical step. The comparative evaluation was

performed by analysis using nano-electrospray ionization mass spectrometry (nano-ESI-MS).

Example

**Patient Sample**

From the Department for General Surgery of the University Clinic at the University of Tübingen, a sample was obtained from a patient who had been histologically confirmed to have intestinal cancer. The patient (referred to as CCA129 in the following) had the HLA class I type HLA-A*01, HLA-A*68, HLA-B*08, HLA-B*44.

**Cell Line**

The Awells cell line was used (European Collection of Cell Cultures, Porton Down, Salisbury, United Kingdom), which has the HLA Classe I Type HLA-A*02, HLA-B*44.

**Keratin 18 Transfected Cell Line**

The Awells cell line was stably transfected with the DNA sequence for human keratin 18 according to standard protocols. For this purpose, human cDNA coding Keratin 18 was subcloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany). The subsequent cloning was done between the restriction endonuclease interfaces EcoR I and Not I of the plasmid vector pcDNA3-Ii in the reading frame of the Ii-sequence. The transfection of the Awells cells was done using electroporation, and subsequently, stable transfectants were selected and kept in culture.

**Isolation of HLA class I-bound peptides**

The preparation of the tissue sample which was shock frozen in liquid nitrogen after being surgically removed was performed as described by Schirle et al., Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach, 2000, European Journal of Immunology, 30:2216-2225. The peptides were isolated according to Standard

Protocols, specifically using the monoclonal antibody W6/32, which is specific for the HLA Class I molecule. Barnstable et al., Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens - new tools for genetic analysis, 1978, Cell, 14:9-20, describes the production and use of this antibody.

**Acetylation of Peptides**

10µl $^{1}H_6$-acetyl anhydride or $^{2}D_6$-acetyl anhydride (50% solution by volume in methanol) was added to 100µl peptide mixture (peptide quantities in mixtures: between 2 nmol and 200 pmol) in a 50% methanol/water mixture (by volume). The chemical reaction occurred for 15 minutes at room temperature. The reaction was stopped by adding 1.1 µl formic acid. Then, the same volume was taken from both approaches and mixed together.

**Guadinylation of Peptides**

Peptide mixtures from tumor tissue (CCA129), or Keratin 18 transfected or not-transfected Awells-cells (peptide quantities in mixtures: between 2 nmol and 200 pmol) in citrate buffer (50 mM citrate, pH 3.0) was mixed with 0.25 % trifluoroacetic acid (TFA, by volume), then the pH of the mixture was adjusted to 10.5 with 200 µl sodium hydroxide (10 M solution). After adding 1 ml O-methylisourea hemisulfate solution (2.5 M in water), the reaction mixture was incubated for 10 minutes at 65°C (water bath). The reaction was stopped by adding 200 µl formic acid.

**Nicotinylation of Guadinylated Peptides**

The peptide mixtures, chemically modified by guadinylation, from tumor tissue (CCA129), or keratin-18-transfected or non-transfected Awells cells were put on a chromatography column of the type "reversed phase C-18 microcolumn" (AgilentTechnologies hydrophobic XGSXB) and washed with 0.5 ml water. Peptides bound to the column material were then left on the column and nicotinylated by slowly applying 1 ml of freshly prepared $^{1}H_4$- or $^{2}D_4$-nicotinyl-N-hydroxysuccini-mid-ester (sodium phosphate buffer 50 mM; pH 8.5) by chemical reaction at room temperature. Next, for a second time, 1 ml of freshly prepared $^{1}H_4$- or $^{2}D_4$-nicotinyl-N-hydroxysuccinimide ester was slowly conducted through the chromatography column loaded with the peptide mixture. After that, hydroxylamine is

guided through the column in order to again remove undesired modifications of tyrosin residues by nicotinyl groups. Then, the chromatography column is washed with water before the peptides are eluted from the column with 100 µl of a 50% acetonitrile/water mixture (by volume).

**Offline-High Performance Liquid Chromatography (HPLC) Separation of peptide mixtures**

Mixtures of peptides chemically modified in this way were mixed in equimolar ratios und contracted in volume to approx. 100 µl by vacuum centrifugation. The contracted mixtures were diluted with 400 µl of water with 0.08 % TFA (by volume), before they were put by automated sample injection onto a "reversed phase"-chromatography column, model µRP SC C2/C18, 100 mm x 2.1 mm (Amersham-Pharmacia, Freiburg, Germany) which was connected to a SMART-HPLC-System (Amersham-Pharmacia, Freiburg, Germany). For chromatographic separation and the elution of the peptides bound to the column material, a binary gradient made from two solvent mixtures A and B was used. Solvent mixture A contains 0.1 % TFA (by volume) in water. Solvent mixture B contains 0.08 % TFA and 80 % acetonitrile (both by volume) in water. The binary gradient begins with 90 % solvent mixture A and 10 % solvent mixture B and follows a linear progression until a mixture ratio of 40 % solvent mixture A and 60 % solvent mixture B. The eluate is collected in fractions with a volume of 150 µl per fraction. Prior to beginning the mass spectrometric studies of the chromatographically separated peptides, the collected fractions are completely dried by vacuum centrifugation and then dissolved again in a mixture of 50 % methanol, 49.9 % water and 0.1 % formic acid.

**Microcapillary Liquid Chromatrography Mass Spectrometry**

The peptide mixtures were analyzed using a reversed-phase-HPLC-Systems ("reversed phase Ultimate HPLC System, Dionex, Amsterdam, Niederlande) connected to a hybrid quadropole mass spectrometry device ("orthogonal acceleration time of flight mass spectrometer", Micromass, Manchester, United Kingdom) equipped with a micro-electrospray ionisation source. For this purpose, the sample material was first desalinated and preconcentrated on a C18-precolumn with the dimensions 300 µm x 5 mm (LC Packings, Amsterdam, Netherlands). A syringe pump (Harvard Apparatus, Inc.) equipped with a gastight 100 µl-

syringe (1710 RNR, Hamilton) delivered solvent and sample at a rate of 2 µl per minute. The precolumn loaded with the peptide mixture is then connected in the flow direction in front of a silica column (75 µm x 250 mm, Dionex, Amsterdam, Netherlands) that is connected to a "reversed phase Ultimate"-HPLC-System and loaded with C18-reversed-phase material (5 µm, Dionex, Amsterdam, Netherlands). For the elution of the bound peptides, a binary gradient is planned over a time period of 120 minutes, which begins with 15 % solvent A (4 mM ammonium acetate in water, pH 3.0) and 85 % solvent B (2 mM ammonium acetate in a mixture by volume from 80 % acetonitrile and 20 % water, pH 3.0) and leads to a mixture ratio of 40 % solvent A and 60 % solvent B. The flow-through rate during the elution of the peptides is reduced by the Ultimate split-System (Dionex, Amsterdam, Netherlands) to ca. 300 µl per minute. The eluate was introduced into the micro-ESI-source through a gold-coated glass capillary (PicoTip, New Objective, Cambridge/Massachusetts, U.S.A.). The integration time for the "time of flight" analysis (TOF analyzer) was set to 1 second, the delay time between two analysis operations was 1/10 second. The ratio of chemically modified peptides with deuterium (2D) atoms to peptides having the same basic amino acid sequence with normal hydrogen (1H) atoms was determined by comparing the relative height of the "peaks" (measured vertex points of the predominate signal from the mass spectrometric analysis).

The online fragmentation of peptides to determine the amino acid sequence (HPLC-MSMS) was performed with an integration time of 4 seconds for the "time of flight" analysis (TOF analyzer) and a delay time of 1/10 second between two analysis operations, and otherwise as described. In the process of the online fragmentation of the $[M + H]^+$ and $[M + H]^{2+}$ ions, a change is made automatically between the HPLC-MS mode and HPLC-MSMS mode. The spectra that emerged from the mass spectrometric analyses were analyzed manually. Data base searches (NCBInr, EST) were performed using MASCOT (http://www.matrixscience.com).

In an additional preferred embodiment for small sample volumes, instead of an HPLC system, metal-coated glass capillaries (Proxeon, Odense, Denmark) could also be used for further reduction of the flow rate in bringing the sample to the micro-ESI-source. In this way, flow rates of 20 nl per minute up to 50 nl per minute are possible. In this embodiment form, the ratio of chemically modified peptides with deuterium ($^2$D) atoms to peptides having the same basic amino acid sequence with normal hydrogen ($^1$H) atoms was determined by comparing the relative height of the "peaks" (measured vertex points of the predominate

signals from the mass spectrometric analysis) and the relative mathematically integrated areas of the "peaks". The fragmentation of peptides in HPLC-MSMS mode is also possible in this embodiment form. This is performed with collision energies of 30-60 eV for $[M+H]^+$-ionized fragments and 20-30 eV for $[M+H]^{2+}$-ionized fragments. For this embodiment form, the integration time for the "time of flight" analysis (TOF analyzer) is 1 second, and between two analysis operations, there is a delay time of 1/10 of a second.

Results

**Fig. 1** depicts the basic principle for differential measurement and identification of MHC-class I bound peptides. In this method, peptides from two different sources are treated with reactive chemical groups which can be distinguished by the presence or absence of certain hydrogen species (light hydrogen: $^1H$; heavy hydrogen: $^2D$), without the physical properties, which are achieved by the different hydrogen isotopes and which are used for differentiation, having a measurable influence on the chemical properties of the modified peptides. The peptide derivatives occurring as a result of the chemical modification are combined with each other and separated by chromatography ("offline"-HPLC or "online"-HPLC-MS) according to their hydrophobicity, or respective hydrophilicity, based on the primary amino acid sequence. The signal intensity of the specific mass/charge signals determined by the subsequent mass spectrometric analysis is the indicator for the relative quantitative ratio between peptides which have the same basic primary amino acid sequence and which were obtained from different sources. The use of the tandem-MSMS method provides additional information, using databases, about the amino acid sequence of the peptides present in the individual case.

The acetylation of MHC class I ligands represents a fast and simple method for the chemical modification of peptides. The acetylation of peptides was optimized experimentally using synthetic peptide mixtures. After a 15 minute reaction time (as previously described), the peptides were acetylated completely on the amino-terminal end.

**The use of $^2D_6$- and $^1H_6$-acetanhydride for the acetylation makes possible the differential quantification of peptides.**

In order to demonstrate feasibility, MHC class I bound peptides were obtained from MGAR cells as described above, separated into two equal-volume partial samples and acetylated as

described with $^{2}D_{6}$-acetanhydride, or respectively $^{1}H_{6}$-acetanhydride. After the end of the chemical reaction, the partial samples were again mixed in equimolar ratios and the relative ratios between $^{2}D_{3}$- and $^{1}H_{3}$-acetylated peptides were determined. **Fig. 2** shows, for example, the $^{2}D_{3}$- and $^{1}H_{3}$-variants of a peptide with the amino acid sequence EVNGLISMY (molecular weight without chemical modification: 1040.5 Da). The peptide EVNGLISMY constitutes a fragment from the "U5 snRNP-specific Protein". The measured relative ratio between $^{2}D_{3}$- and $^{1}H_{3}$-variants ($^{2}D_{3}/^{1}H_{3}$-ratio) of EVNGLISMY was 1.0. For 15 additional peptides, which had been eluted from the same MGAR cells and had been detected by mass spectrometry as singly or doubly charged ions, the $^{2}D_{3}/^{1}H_{3}$-ratio had an arithmetic mean of 1.01. The standard deviation (SD) was ±0.13 (Table 1). In addition to making possible a determination of the relative portions of peptides with the same basic amino acid sequence from two or more different sources, the acetylation of peptides by the shifting of the b-series-ions by 3 Da of $^{2}D_{3}$-acetylated relative to $^{1}H_{3}$-acetylated peptides also leads to a simplification of the valuation of the corresponding mass spectrogram (Fig. 2C). b-series ions are generally ionized fragments of both chemically modified as well as non-modified peptides which contain at least the amino acid residue placed at the amino terminus in the amino acid primary sequence. y-series ions are, as opposed to b-series ions, generally ionized fragments of both chemically modified, as well as non-modified peptides, which contain at least the amino acid residue placed at the carboxyl terminus in the amino acid primary sequence.

It is disadvantageous in the acetylation of peptides that the ionization by the introduction of the acetyl residue onto the amino-terminus end of the peptides can cause a positive charge less than in a peptide with an intact N-terminus. Since in principle, multiply charged peptides can be better detected by mass spectrometry than singly charged peptides, the acetylation also causes a loss of sensitivity. The experiments performed also showed that acetylation of the ε-amino group of lysine residues can occur. This acetylation of the ε-amino group of lysine residues also has the result that by the ionization, a positive charge less than in the peptide not chemically modified by acetylation can occur. The resultant loss in sensitivity, however, applies to the same extent for the basic peptides of the same amino acid primary sequence from the differently used sources, so that a measurable effect on the inner ratio between the associated signals of the equivalently sequenced, in one case $^{2}D_{3}$-acetylated peptides and in the other case $^{1}H_{3}$-acetylated peptides, does not occur.

**Identification of MHC class I-bound peptides from tissue samples of intestinal tumors and**

**tissue samples of normal tissue surrounding the tumors using $^2D_3$- and** $^1H_3$-acetylation of the MHC class I-bound peptides in a mass spectrometric analysis comparing the relative quantities based on electrospray ionization mass spectrometry (ESI-MS). P eptides were isolated as described from MHC classe I molecules of an intestinal cancer sample (CCA129) and from MHC class I molecules of a sample of the normal tissue surrounding the surgically removed tumor and then chemically modified by $^2D_3$- (tumor) and $^1H_3$-(normal tissue) acetylation. After the chromatographic separation of the modified peptides by microbore-HPLC, 19 peptides were identified as described by nano-ESI-MS. F or 17 of these 19 peptides, the relative quantitative ratio could be determined by a comparison of the specific peptides of the tumor tissue sample to the peptides of the sample of normal tissue with the same basic amino acid sequence. The majority of the identified peptides was present in similar quantities in both the samples examined ($^2D_3/^1H_3$ ratios between 1.07 and 2.42). On the whole, a 1.7 times greater quantity of peptides was present in the tumor sample compared to the normal tissue sample. Two peptides were overrepresented in the tumor tissue, and one peptide was underrepresented in the tumor. The statistical evaluation of the results using the "student's t-test" confirmed that only the two over-expressed and the one under-expressed peptide was outside a 99.99% confidence intervall of 0.87 to 2.56.

The two peptides over-represented in the tumor derived from the human proteins "ribosomal protein L24" and beta-catenin. While little data with regard to a tumor-association exists for the ribosomal protein L24, a role in the origination of esophageal cancer was described for the "ribosomal protein L15", which is related to the "ribosomal protein L24", by Wang et al., 2001, Cloning and characterization of full-length human ribosomal protein L15 cDNA which was overexpressed in esophageal cancer, Gene 263:205-209. For beta-catenin, on the other hand, a function as an oncogen, which turns on by transactivation of other oncogens, such as, for example, the matrix metalloproteinase MMP-7, was described by Ougolkov et al., 2002, Oncogenic beta-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer, Gastroenterology 122: 60-71. A mutated beta-catenin peptide was described by Robbins et al., 1996, A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor-infiltrating lymphocytes, Journal of Experimental Medicine 183:1185-1192, as a target structure in connection with the human MHC-Allel HLA-A*24 for CD8-positive, skin cancer-infiltrating T cells.

Improved yield of MHC classe I-bound peptides after chemical modification of the peptides

by O-methylisourea-hemisulfate and nicotinyl-N-hydroxy-succinimide ester (NicNHS). The initial and new combination of two methods for chemical modification of peptides by combination of the einheitlichen guadinylation of ε-amino groups of lysin residues in peptides by O-methylisourea-hemisulfate and the nicotinylation of α-amino groups of peptides by NicNHS leads to a clear improvement of the ionization of peptides (***Fig. 4***). In order to simplify the desalination of the chemically modified peptides, the nicotinylation of the peptides is performed on a C18-chromatography column as described above. The undesired modification of the side-chains of tyrosine residues induced by the nicotinylation could be removed again by treatment of the modified peptides with hydroxylamine. For example, it is shown using the peptide with the amino acid sequence AETSYVKL in **Fig. 4**, that the nicotinylation of the N-terminus affects the ionization in such a way that the nicotinylated peptides can be detected as well as the peptides that have not been chemically modified.

**Identification and Quantification of MHC class I bound peptides from the Awells cell line and the Awells cell line transfected with a plasmid containing the cDNA of the human keratin 18 by guadinylation and $^{2}D_{4}$-/$^{1}H_{4}$-nicotinylation of the peptides.** In Trask et al., 1990, Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells, Proc. Natl. Acad. Sei. U.S.A., 87:2319-2323, it was shown that keratines are suitable as markers for distinguishing tumor and normal tissue. In order to identify new MHC classe I-bound peptides from human keratin 18 and to demonstrate the differential quantification using an exemplary tumor antigen, peptides were isolated from the non-transfected (Awells) and from the Awells cell line transfected with the named plasmid (Awells keratin 18). The isolated peptide mixtures were then chemically modified as described by guadinylation and $^{2}D_{3}$-, respectively $^{1}H_{3}$-nicotinylation. The chemically modified peptide mixtures were mixed together and examined by HPLC-MS analysis as described. In a second experiment, work was done in MSMS mode, so that the amino acid sequences of in total 27 different peptides could be determined. All 27 peptides found, with the exception of a peptide with a molecular weight of 1091.6 Da were both transfected as well as detected on non-transfected cells in quantities which were within the confidence interval of 0.64 to 2.28 (statistical evaluation using the "Student's t-test"). For the peptide with a molecular weight of 1091.6 Da, the amino acid sequence RLASYLDRV was determined using MSMS analysis, which is a fragment of the amino acid sequence of keratin 18. The MSMS spectra, which led to identification of the peptide with the sequence RLASYLDRV, are shown in **Fig.** 3D. For

the peptide RLASYLDRV, no signal could be detected which could be linked to a chemical modification of the primary sequence with a $^{1}H_3$-nicotinyl residue. This observation suggests that keratin 18 was expressed exclusively in the Awells keratin 18 cells. The signal for the peptide RLASYLDRV with a $^{2}D_3$-nicotinyl residue, on the other hand, was expressed six times greater than the background.

The described method of guadinylation and nicotinylation of peptides using the two hydrogen isotopes $^{1}H$ and $^{2}D$ allows for the first time the fast and exact determination of relative quantitative differences between sequence-equivalent peptides from two or more different sources. By the use of the method for guadinylation and nicotinylation of peptides on samples of tumor tissue and normal tissue of the same organ, or by the use of the mentioned method on cell lines which had been transfected before by nucleic acids coding for oncogenes or other tumor-associated gene products, tumor-associated peptide antigens can be determined which are especially suitable for the production of vaccines for cancer treatment.

The results described above are summarized in Tables 1 to 3, the sequence protocol shows the peptides according to the invention.

# Sequence listing

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<120> Method for identification and quantification of tumor-associated peptides

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# Tables

**Table 1** shows the measured values of mass spectrometric signal intensities from an equimolar mixture of $^{2}D_3$-nicotinylated peptides to $^{1}H_3$-nicotinylated peptides from MGAR-cells and the derived ratios of $^{2}D_3$-nicotinylated peptides to $^{1}H_3$-nicotinylated peptides each with the respective same basic amino acid sequences. Peptides with double positive charge $[M+H]^{2+}$ have a dramatically higher signal intensity than peptides with a single positive charge $[M+H]^{+}$. Peptide ions with double positive charge $[M + H]^{2+}$ have different basic sequences than peptides with a single positive charge $[M + H]^{+}$.

| **Peptide Ionization condition** | **m/z-ratio $^{1}H_3$-acetylated peptide ions** | **$^{1}H_3$ peptide signals [counted ions]** | **$^{2}D_3$- peptide signals [counted ions]** | **$^{2}D_3/^{1}H_3$-Ratio** |
|---|---|---|---|---|
| $[M+2H]^{2+}$ | 554.45 | 409 | 361 | 0.88 |
| | 567.99 | 590 | 511 | 0.86 |
| | 574.50 | 316 | 306 | 0.96 |
| | 578.52 | 884 | 877 | 0.99 |
| | 582.01 | 615 | 598 | 0.97 |
| | 597.96 | 612 | 800 | 1.30 |
| | 604.95 | 499 | 558 | 1.11 |
| | 612.49 | 515 | 502 | 0.97 |
| $[M+H]^{+}$ | 941.49 | 33 | 32 | 0.97 |
| | 992.51 | 39 | 51 | 1.30 |
| | 1028.55 | 23 | 20 | 0.87 |
| | 1039.47 | 21 | 22 | 1.04 |
| | 1057.56 | 30 | 32 | 1.06 |
| | 1083.48 | 63 | 63 | 1.00 |
| | 1092.47 | 59 | 54 | 0.92 |
| | 1170.47 | 77 | 70 | 0.90 |
| | **Mean value** | | | **1.01** |
| | **Standard deviation** | | | **±0.13** |

**Table 2:** Table 2 shows sequences and measurement results for natural peptides obtained from HLA class I molecules from tumor tissue and normal tissue of a patient with intestinal cancer. To perform the analysis, the peptides isolated from the tissue samples were chemically modified with $^{2}D_{6}$-acetic anhydride (tumor tissue) or $^{1}H_{6}$-acetic anhydride (normal tissue), mixed, identified according to the method by mass spectrometric analysis, and the quantitative ratios occurring between the tissue samples, of peptides with identical amino acid sequences were quantified by determining the $^{2}D_{3}/^{1}H_{3}$ ratio. In order to specify significant over-representation and/or under-representation of identified and quantified peptides, the measurement results ($^{2}D_{3}/^{1}H_{3}$ ratio) were evaluated by statistical analysis (Student's t-test).

| Peptide Sequence (HLA anchor residues in bold) | HLA | Protein source | AA-Position | Ratio $^{2}D_{3}/^{1}H_{3}$ |
|---|---|---|---|---|
| T T E Q H G AR **Y** | **A*01** | Tapasin | **372-380** | **n.d.** |
| F T **K** V**K** P L **L** | **B*08** | Myosin heavy chain, nonmuscle A | **831-838** | **n.d.** |
| V**A** V G V A R A **R** | **A*68** | Poly IG receptor | **656-664** | **0.69** |
| D V S H T V V L **R** | **A*68** | Translocon-associated protein ß-SU | **88-96** | **1.07** |
| T **L** G D I V F K **R** | **A*68** | Fatty acid-bindin protein, liver | **114-122** | **1.14** |
| D I **H** H**K** V L S **L** | **B*08** | Ras-GAP SH3 binding protein 2 | **60-68** | **1.30** |
| E **V** T R I L D G **K** | **A*68** | SH3BGR3-!ike protein | **23-31** | **1.32** |
| R **V** A P E E H P V **L** | **n.a.** | Actin, cytoplasmic 1 | **94-103** | **1.39** |
| T **T** A E R E I V **R** | **A*68** | Actin alpha | **204-212** | **1.45** |
| S **I** F D G R V V A**K** | **A*68** | Putative membrane protein (PNASIO) | **88-97** | **1.45** |
| E **A** G P S I V H **R** | **A*68** | Actin alpha | **366-374** | **1.51** |
| D**T** A A Q I T Q **R** | **A*68** | MHC class I antigen (HLA-B) | **136-144** | **1.61** |
| D**T** I E I I T D **R** | **A*68** | HNRPA2/BI | **139-147** | **1.66** |
| E **S** T G S I A K **R** | **A*68** | AldolaseA | **34-42** | **1.73** |
| A **V** A A V A A R **R** | **A*68** | Glucosidase II alpha subunit | **3-11** | **1.91** |
| T **A** A D T A A Q I T **R** | **A*68** | MHC class I antigen (HLA-B) | **133-144** | **2.19** |
| E **S** G **P** S I V H **R** | **A*68** | Actin beta | **364-372** | **2.42** |
| D**A** A H P T N V Q **R** | **A*68** | Beta-catenin | **115-124** | **3.02** |
| S **L** A D I M A K **R** | **A*68** | Ribosomal protein L24 | **86-94** | **3.27** |
| **Mean value** | | | | **1.71** |
| **Standard deviation** | | | | **±0.68** |
| **Student-t test:** Confidence interval 99.99 % (n= 27) | | | | **0.87-2.56** |

n.a., not assigned; n.d., not determined

**Table 3** shows sequences and measurement results for natural peptides obtained by HLA-class I molecules from the unchanged Awells cell line and the Awells cell line genetically altered as described by transfection with keratin 18. From the two sources named, Awells and Awells keratin 18, isolated peptides were changed by chemical modification with $^{1}H_{4}$-nicotinyl residues (Awells) or $^{2}D_{4}$-nicotinyl residues (Awells keratin 18). According to the method, peptides were mixed after the end of the chemical modification and identified by mass spectrometric analysis and the

quantitative ratios existing between Awells and Awells keratin 18 of peptides with identical amino acid sequences was quantified by determining the $^2D_4/^1H_4$ ratio. In order to specify significant over-representation and/or under-representation of identified and quantified peptides, the measurement results ($^2D_3/^1H_3$ ratio) were evaluated by statistical analysis (Student's t-test).

| **Peptide Sequence (HLA anchor residues in bold)** | **HLA** | **Source protein** | **AA-Position** | **Ratio ($^2D_4/^1H_4$)** |
|---|---|---|---|---|
| K **E** S T L H L V **L** | B*44 | Ubiquitin | 63-71 | 0.96 |
| AE S L L T M E **Y** | B*44 | KIAA1390 | 31-39 | 1.02 |
| L **L** M E H T M V A **F** | A*02 | EST | 24-33 | 1.05 |
| HL A VE R G K **V** | A*02 | Similartox-kinase | 532-540 | 1.06 |
| S **E** I E A K VR **Y** | B*44 | Talin I | 290-298 | 1.08 |
| T **L** F P G K VH S **L** | A*02 | WD-repeat protein 6 | 432-441 | 1.09 |
| S **E** D N R I L L **W** | B*44 | Methylosome protein 50 | 187-195 | 1.10 |
| S **I** I G R L L E **V** | A*02 | PhosphatasePPl-al | 11-19 | 1.12 |
| YL L P A I V H **I** | A*02 | RNA-dependent helicase p68 | 148-156 | 1.12 |
| QL V D I I E K **V** | A*02 | Proteasome activator complex SU 3 | 114-122 | 1.14 |
| AL L D K L Y A **L** | A*02 | Simiiarto mitochon. ribosom. prot. S4 | 78-86 | 1.14 |
| I **E** H G I I T N **W** | B*44 | Actin alpha skeletal muscle | 73-81 | 1.19 |
| I **M** L E A L E R **V** | A*02 | Small nuclear ribonucleoprotein G | 68-76 | 1.19 |
| L **L** F D R P M H **V** | A*02 | HNRP M | 268-276 | 1.22 |
| AE K L I T Q T **F** | B*44 | NPDOM | 2-10 | 1.23 |
| R **L** A Q H I T Y **V** | A*02 | Licensing factor MCM7 | 532-540 | 1.25 |
| S **E** P D F V A K **F** | B*44 | FLJ2067I | 121-129 | 1.28 |
| T **E** V T G H R **W** | B*44 | Basigin | 48-55 | 1.37 |
| AE T P D I K L **F** | B*44 | 40S ribosomal protein S5 | 12-20 | 1.48 |
| QE H VK S F S **W** | B*44 | Sortilin-related receptor | 245-253 | 1.50 |
| AI V D K VP S **V** | A*02 | Coatomer gamma subunit | 147-155 | 1.52 |
| E **E** P T V I K K **Y** | B*44 | Sorting nexin 5 | 257-265 | 1.54 |
| QE A G I K T A **F** | B*44 | Multifunctional protein ADE2 | 69-77 | 1.60 |
| GE A S L R L A H **Y** | B*44 | HistoneH2B.f | 75-83 | 1.65 |
| QE D L R T F S **W** | B*44 | Ras-GTPase-activating protein | 243-251 | 1.66 |
| M **E** Q V I F K Y **L** | B*44 | Actin-like protein 3 | 93-101 | 1.98 |
| R **L** A S Y L D R **V** | A*02 | Cytokeratin 18 | 89-97 | ≥5.95 |
| **Mean value** | | | | **1.46** |
| **Standard deviation** | | | | **±0.93** |
| **Student-t test:** Confidence interval 99.99 % (n=27) | | | | **0.64-2.28** |

## Patent Claims

1. Method for identification and quantification of tumor-associated peptides, with the steps:

   a) Preparing a sample from tumorous tissue and a sample from corresponding normal tissue or correspondingly transfected cell lines, whereby both samples have the same quantities by weight or cell numbers,
   b) Isolation of peptides from the sample made from tumorous tissue,
   c) Isolation of peptides from the sample made from the corresponding normal tissue,
   d) Chemical change of the peptides obtained from step (b) with a chemical group that contains a stable isotope of an element from the Periodic Table of the Elements,
   e) Chemical change of the peptides obtained from step (c) with a chemical group that contains a second stable isotope of the element used in step d) from the Periodic Table of the Elements, whereby the stable isotope used in this step is lighter or heavier than the isotope used in step d),
   f) Mixing of the chemically modified peptides obtained from steps (d) and (e),
   g) Separation of the peptides obtained from step f) by a chromatographic process,
   h) Identification and determining of peptides with identical amino acid sequences and the quantitative ratios of the chemically modified peptides with identical amino acid sequences from step (g), i) Identification of tumor-associated peptides suitable for the composition of a therapeutic vaccine based on the data obtained from step (h).

2. Method according to claim 1, characterized in that the tumor-associated peptides are MHC class I ligands.

3. Method according to claim 1 or 2, characterized in that in step d) deuterium ($^2$D) and in step e) normal hydrogen ($^1$H) are used as stable isotopes.

4. Method according to one of the claims 1 to 3, characterized in that in step g) the chromatographic separation of the peptides by HPLC is performed.

5. Method according to one of the claims 1 to 4, characterized in that the step h) is done by mass spectrometric analysis.

6. Method according to claim 5, characterized in that the mass spectrometric analysis for

determining the quantitative ratios of two peptides with identical amino acid sequence and identical chemical modification is performed using at least two different isotopes of the same element from the Periodic Table of the Elements.

7. Method according to claim 6, characterized in that the relative intensity of measured signals for peptides of identical amino acid sequences and identical chemical modification with simultaneously present different isotopes of the same element serves to calculate the relative quantitative ratios between the named peptides.

8. The method according to one of the claims 1 to 7, characterized in that the presence and/or the quantitative ratio of a peptide can be used as a diagnostic marker.

9. Method according to one of the claims 1 to 8, characterized in that in step i) suitable databases for identifying tumor-associated genes and gene products are used.

10. Method according to claim 1 to 9, characterized in that after step h) a further step is performed, in which the reactivity of peripheral leukocytes, preferably T-lymphocytes, is tested against the identified and quantified peptides.

11. Method according to claim 10, characterized in that the reactivity test is performed by means of measuring cytokine-mRNA and/or interferon mRNA synthesized by the leukocytes.

12. Method according to claim 10, characterized in that the test of the reactivity is done by the activation of peripheral T-lymphocytes by means of reconstituted complexes made from molecules and peptides presenting antigens.

13. Method according to claim 12, characterized in that the complexes used for activating T-lymphocytes from antigen-presenting molecules and peptides are fixed on a surface.

14. Method according to claim 13, characterized in that the surface used for fixing the complexes made from the molecules and peptides presenting antigens consists of polystyrene.

15. Method according to one of the claims 12 to 14, characterized in that the molecules

presenting antigens are connected to biotin by a chemical reaction and the surface used is made of polystyrene coated by chemical reaction with streptavidin.

16. Method for producing a peptide, in which according to the method according to one of the claims 1 to 15, a peptide is identified and the identified peptide is synthesized chemically, in vitro, or in vivo.

17. Peptide which was identified by a method according to one of the claims 1 to 15 and/or produced by a method according to claim 16.

18. Pharmaceutical composition containing one or more peptides according to claim 17.

19. Use of the peptide according to claim 17 or the composition according to claim 18 for treatment of tumor disease.

20. Use of the peptide according to claim 17 for the production of an agent for treating tumor diseases

21. Use of the peptide according to claim 17 to evaluate a treatment progression of a tumor disease.

22. Nucleic acid molecule coding for a peptide according to claim 17.

23. Vector, containing the nucleic acid molecule according to claim 22.

24. Cell that has been genetically modified in such a way using a nucleic acid molecule according to claim 22 that it produces the peptide according to claim 17.

25. Use of the peptide according to claim 17 to produce an antibody.

26. Method for producing a vaccine with the steps:

a) Performing the method according to one of the claims 1 to 15,

b) production of the identified peptides, and

c) formulation of the peptides produced into the vaccine.

27. Tumor-associated peptide with an amino acid sequence chosen from the group consisting of SEQ-ID no. 1 to 36 from the attached sequence protocol, whereby the peptide is capable of binding to a molecule of the human major histocompatibility complex (MHC) class I.

28. Peptide according to claim 27, characterized in that at least one amino acid is replaced by another amino acid with similar chemical properties.

29. Peptide according to claim 27 or 28, characterized in that N-terminus or C-terminus at least one additional amino acid is present.

30. Peptide according to one of the claims 27 to 29 characterized in that at least one amino acid is deleted.

31. Peptide according to one of the claims 27 to 30 characterized in that at least one amino acid is chemically modified.

32. Use of one or more of the peptides according to one of the claims 27 to 31 for producing a drug for treatment of tumor diseases and/or adenomatous diseases.

33. Use of the peptide according to one of the claims 27 to 31 for treatment of tumor diseases and/or adenomatous diseases.

34. Use according to claim 32 or 33, characterized in that the disease is renal, lung, intestinal, gastric, pancreatic, breast, prostate, ovarial and/or skin cancer.

35. Use according to one of the claims 32 to 34, characterized in that the peptide is used together with an adjuvant.

36. Use according to one of the claims 32 to 35, characterized in that a peptide bound to a cell presenting an antigen is used.

37. Use of the peptide according to one of the claims 27 to 31 for marking leukocytes, in particular T-lymphocytes.

38. Use of the peptide according to one of the claims 27 to 31 to evaluate a therapy progression for a tumor disease.

39. Use of a peptide according to one of the claims 27 to 31 to produce an antibody.

40. Pharmaceutical composition containing one or more of the peptides according to one of the claims 27 to 31.

41. Nucleic acid molecule coding for the peptide according to one of the claims 27 to 31.

42. Vector, containing the nucleic acid molecule according to claim 41.

43. Cell which, using the nucleic acid molecule according to claim 41, or with the vector according to claim 42, was genetically changed in such a way that it expresses a peptide according to one of the claims 27 to 31.

44. Method of identifying and quantifying tumor-associated peptides in which at first at least two different sources (tumor and normal tissue or correspondingly transfected cell lines), of the same quantity by weight or cell number, are prepared for extraction of the peptides, and the peptides from the different sources are then chemically modified in an identical manner separately from each other using at least two different stable isotopes of the same element, the peptides modified in this way are then mixed and after that preferably isolated by chromatographic methods and the amino acid sequences of the peptides are determined, so that the determination of the relative quantitative ratios of sequence-identical peptides from different samples to each other is done using the stable isotopes used in the chemical modification.

Abstract

The invention relates to a method for identifying and quantifying tumor-associated peptides whereby initially at least two different sources are prepared for extracting peptides (tumor and healthy tissue) and the peptides from the different sources are chemically modified separately from each other in an identical manner using at least two different stable isotopes of the same element. Next, the peptides are isolated by a chromatographic process and the amino acid sequences of the peptides are determined, whereby the measurement of the relative quantitative ratios of sequence-identical peptides from different samples to one another is done by means of the stable isotopes that are used in the chemical modification. Furthermore, the invention involves a tumor-associated peptide with an amino acid sequence chosen from the group consisting of SEQ-ID no. 1 to 36 from the attached sequence protocol, whereby the peptide is capable of binding to a molecule of the human major histocompatibility complex (MHC) class I. The invention involves, furthermore, the use of peptides for manufacturing a drug and for treating tumor diseases and/or adenomatous diseases. In addition, a pharmaceutical composition is described which has at least one of the peptides.

**Abstract:**
The invention relates to a method for identifying and quantifying tumor-associated peptides whereby initially at least two different sources are prepared for extracting peptides (tumor and healthy tissue) and the peptides from the different sources are chemically modified separately from each other in an identical manner using at least two different stable isotopes of the same element. Next, the peptides are isolated by a chromatographic process and the amino acid sequences of the peptides are determined, whereby the measurement of the relative quantitative ratios of sequence-identical peptides from different samples to one another is done by means of the stable isotopes that are used in the chemical modification. Furthermore, the invention involves a tumor-associated peptide with an amino acid sequence chosen from the group consisting of SEQ-ID no. 1 to 36 from the attached sequence protocol, whereby the peptide is capable of binding to a molecule of the human major histocompatibility complex (MHC) class I. The invention involves, furthermore, the use of peptides for manufacturing a drug and for treating tumor diseases and/or adenomatous diseases. In addition, a pharmaceutical composition is described which has at least one of the peptides.

## Description

The invention relates to a method for identifying and quantifying tumor-associated peptides and to a method for preparing tumor-associated peptides and to the peptides identified/quantified/prepared thereby as well as their use.

Such peptides are, for example, used in the immunotherapy of tumor diseases.

The recognition of tumor-associated antigens (TAA) by immune system components plays a major role in the elimination of tumor cells by the immune system. This mechanism is based upon the fact that there exist qualitative or quantitative differences between tumor cells and normal cells. In order to generate an immune system response directed at the tumor, the tumor cells must express antigens against which an immune response sufficient to eliminate the tumor can be induced.

CD8-expressing cytotoxic T-lymphocytes (hereinafter referred to as CTL) play a large role in the elimination of tumors. To trigger an immune reaction of this type by CTL, foreign proteins/peptides must be presented to the CTL. T cells recognize antigens as peptide fragments only if they are presented on cell surfaces by MHC ("major histocompatibility complex") molecules. These MHC molecules are peptide receptors that normally bind peptides within the cell in order to transport them to the cell surface. This peptide/MHC molecule complex can be recognized by T cells. Human MHC-molecules are also called human leukocyte antigens (HLA).

In the past, antigen-specific immunotherapy based on T-cells has proven successful in the treatment of cancer.

Induction of a specific CTL response directed against a tumor is dependent on identification of MHC class I-ligands derived from tumor-associated antigens (TAA). Such tumor-associated antigens can be exclusively present in malignant cells, for example as products of mutated genes. Other important classes of tumor-associated antigens are tissue-specific structures such as melanocyte-differentiating antigens. A third class of tumor-associated antigens are proteins that are overexpressed in tumors.

### State of the Art

The methods for identification and characterization of TAA, which represent the starting point for a therapeutic vaccine, are - on the one hand - based on the stimulation of CTL or antibodies that are already present in the patient. This immunological approach is combined either with an analysis of the gene expression profile or with a mass-spectrometry (MS) sequencing of the identified genes (see van der Bruggen, et al., 1991, A gene encoding an antigen recognized by cytolytic T-lymphocytes on a human melanoma, Science 254: 1643-1647, and Cox et al., 1994, Identification of a peptide recognized by five melanoma-specific human cytotoxic T-cell lines, Science 264:716-719). Methods for identifying TAA, which are based on the comparative analysis of the transcription profile of tumor tissue and normal tissue, are, for example, the use of traditional DNA chip technology and processes for the hybridization of messenger-RNA from the tissue samples to be compared.

Celis et al., 1994, Induction of anti-tumor cytotoxic T-lymphocytes in normal humans using primary cultures and synthetic peptide epitopes, Proc. Natl. Acad. Sci. USA 91: 2105-2109, applied a method which takes advantage of the prediction of MHC class I-ligands derived from a selected TAA, and in which these ligands were verified experimentally as T-cell epitopes in a next step.

It is disadvantageous in this procedural method, which necessitates the availability of T-cells from patients as a prerequisite, that the experimental use and cultivation is very expensive.

Schirle et al., 2000, Identification of tumor-associated MHC class I ligands by a novel T-cell independent approach, Eur. J. Immunol. 30:2216-2225, describe a method not dependent on T-cells in which the prediction of MHC class I ligands is combined with the intended search for the predicted peptide ligands in complex peptide mixtures, whereby the peptides were identified by the combination of highly sensitive capillary liquid chromatogrphy with mass spectroscopy (LC-MS).

See for example Young et al., 2001, Expression profiling of renal epithelial neoplasms: a method for tumor classification and discovery of molecular markers, Am. J. Pathol., 158:1639-1651, show that using analyses with DNA chip technology, a large portion of TAA from individual tumors can be identified. MHC class I ligands derived from overexpressed, selectively or exclusively expressed proteins provide potential targets for a CTL-based elimination of tumors. Mathiassen et al, 2001, Tumor-associated antigens identified by mRNA expression profiling induce protective anti-tumor immunity, Eur. J. Immuno. 31: 1239-1246, demonstrated, in a mouse model, that by combining gene expression analysis with epitope prediction, a successful vaccine can be prepared.

The disadvantage of the epitope prediction is due to the fact that for a small number of TAA, a very large number of possible MHC class I ligands is specified, the majority of which is actually not presented by MHC class I molecules at all. This is why the majority of the only predicted epitopes can not induce a CTL-based elimination of tumors.

Weinschenk et al., 2002, Integrated functional genomics approach for the design of patient-individual antitumor vaccines, Cancer Res. 62:5818-5827, show that by the combination of a gene expression analysis with the MHC class I ligands of a tumor, which were isolated and analyzed by liquid chromatography and mass spectroscopy, intended candidates for the composition of a therapeutic vaccine can be determined in one method. The big advantage compared to the exclusive use of gene expression analysis or mass spectroscopy lies in the fact that the MHC class I ligands are analyzed from a complex peptide mixture and they are suitable to a certain extent as immunoreactive peptides due to the fact that they are not only actually presented by MHC class I molecules but also derived from genes which become expressed exclusively, selectively or especially highly in tumors.

## Purpose

The inventors of the application presented here have now recognized that a disadvantage of the combined methods from gene expression analysis and mass spectroscopy consists in not reliably identifying those peptides which induce a CTL response directed against MHC class I ligands of TAA that are presented high on the tumor and in low on the normal tissue. Thus, the purpose of the present invention is to provide a novel method with which in a simple and targeted way, immunoreactive peptides can be identified which induce a CTL response that is directed against MHC class I ligands of TAA that are presented high on the tumor and low on the normal tissue.

The inventors have recognized that this purpose is achieved when for the identified peptides, the quantitative ratios of the peptides actually presented by MHC class I molecules are determined: between the tumor tissue and normal tissue, and/or between correspondingly transfected or infected cells and non-transfected or infected cells.

A method according to the invention for identifying and quantifying tumor-associated peptides thus includes the following steps: Preparing a first sample of tissue or cells, preparing a second sample or tissue or cells with the same quantity by weight and/or cell number as the first sample, extracting peptides from the first and the second sample, separate, chemically identical modifying of the peptides from both samples in order to generate different physical properties in the peptides from the different samples, mixing the peptides that have been modified in this way from both samples, determining the amino acid sequences of the peptides, and determining the relative quantitative ratios of sequence-identical peptides from the two samples based on the different physical properties, whereby preferably the peptides from the two samples are chemically modified by at least two different stable isotopes of the same element.

The purpose is achieved according to the invention by a method of identifying and quantifying tumor-associated peptides in which at first at least two different sources (tumor and normal tissue or correspondingly transfected cell lines), of the same quantity by weight or cell number, are prepared for extraction of the peptides, and the peptides from the different sources are then chemically modified in an identical manner separately from each other using at least two different stable isotopes of the same element, the peptides modified in this way are then mixed and after that preferably isolated by chromatographic methods and the amino acid sequences of the peptides are determined, so that the determination of the relative quantitative ratios of sequence-identical

peptides from different samples to each other is done using the stable isotopes used in the chemical modification.

The peptides are then isolated according to standard protocols, for example, using a monoclonal antibody such as, for example, W6/32, which is specific for the HLR class I molecule.

In order to ensure that the starting material from the two sources has the same quantity (weight in tissue) or cell number, a normalization can also be done using, for example, a peptide uniformly occuring in the tumor tissue and normal tissue, or another marker.

Furthermore, the invention involves a tumor-associated peptide, identified according to the new method, with an amino acid sequence chosen from the group consisting of SEQ-ID no. 1 to 36 from the attached sequence protocol, so that the peptide is capable of binding to a molecule of the human major histocompatibility complex (MHC) class I.

The invention involves, furthermore, the use of peptides or nucleic acid molecules coding for the peptides for manufacturing a drug and for treating tumor diseases and/or adenomatous diseases.

A method according to the invention for identifying and quantifying tumor-associated peptides thus includes the following steps:
a) Preparing a sample from tumorous tissue and a sample from corresponding normal tissue or correspondingly transfected and/or infected cell lines, whereby both samples have the same quantities by weight or cell numbers,
b) Isolation of peptides from the sample made from tumorous tissue,
c) Isolation of peptides from the sample made from the corresponding normal tissue,
d) Chemical change of the peptides obtained from step (b) with a chemical group that contains a stable isotope of an element from the Periodic Table of the Elements (e.g. Deuterium, $^{2}D$),
e) Chemical change of the peptides obtained from step (c) with a chemical group that contains a second stable isotope of an element used in step d) from the Periodic Table of the Elements (e.g. Hydrogen, $^{1}H$),
f) Mixing of the chemically modified peptides obtained from steps (d) and (e),
g) Separation of the peptides obtained from step f) by a chromatographic process,
h) Identification and determination of peptides having identical amino acid sequences and the quantitative ratios of chemically modified peptides with identical amino acid sequences from step (g),
i) Identification of tumor-associated peptides with applicability, preferably excellent applicability for the composition of a therapeutic vaccine based on the data obtained from step (h).

The inventors have recognized that by the method based preferably on mass spectroscopy and on differential chemical modification for determining the differences in the quantitative ratios of peptides between tumor tissue and normal tissue, peptides can be identified that are especially suitable for the composition of therapeutic vaccines.

With the method according to the invention it is thus possible to identify peptides which are suitable for the individual composition, for example, of a personalized mixture of tumor-associated peptides for an individual patient, whereby the peptides can then induce a targeted CTL response adapted to the individual patient's requirement.

For example, industrial laboratories - after having received patient samples - can systematically and efficiently perform this method, and can - after having identified suitable peptides - provide clinics in charge with the peptide sequences; the clinics can then synthesize and formulate the peptides as a therapeutic vaccine. However, it is also possible that a laboratory is carrying out identification as well as preparation, formulation, and production of the tumor-associated peptides suitable for the respective patient.

Also, the systematic and frequent application of the method can lead to a commercial use of suitable tumor-associated peptides, which are found especially frequently as MHC class I ligands, as finished drugs.

The new method is thus applicable both in the context of a pure service as well as in connection with production, formulation and preparation, both for an individual patient, as well as on a suitable industrial scale for use by businesses of the pharmaceutical industry.

In a preferred embodiment, the peptides isolated in steps (b) and (c) are MHC class I ligands.

Only peptides which are bound to MHC-molecules can induce a CTL immune response. Peptides which are derived, for example, from overexpressed genes of a tumor but which are not bound to MHC-molecules, do not induce a CTL immune reaction. Thus, not all peptides, e.g. those peptides only determined by prediction of epitopes, are actually suitable for inducing an immune response.

In an additional preferred embodiment form, step (d) is performed via the guadinylation of the ε-amino group of a lysine residue of a peptide by chemical reaction of peptides with O-methylisourea hemisulfate and the nicotinylation of the α-amino group by chemical reaction of peptides with 2D4-nicotinyl amino hydroxy succinimide (2D4-NicNHS). The guadinylation of the ε-amino group of the lysine residues of peptides is decribed, for example, in Beardsley et al., 2002, Optimization of guadination procedures for MALDI mass mapping, Anal. Chem. 74:1884-1890. The nicotinylation of the a-amino group of peptides is described, for example, in Munchbach et al., 2000, Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation-directing moiety, Anal. Chem. 72:4047-4057.

In an additional preferred embodiment form, step (e) is performed via the guadinylation of the ε-amino group of a lysine residue of a peptide by chemical reaction of peptides with O-methylisourea hemisulfate and the nicotinylation of the α-amino group by chemical reaction of peptides with $^{1}H_4$-nicotinyl amino hydroxy succinimide ($^{1}H_4$-NicNHS).

In an additional preferred embodiment form, the analysis in steps (g) and (h) is done using a coupled liquid chromatography and mass spectroscopy process. Using this technique, the individual chemically modified peptides can be determined exactly and efficiently and with a high throughput. The use of mass spectroscopy to determine chemically modified peptides is, for example, described in Munchbach et al., 2000, Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentationdirecting moiety, Anal. Chem. 72:4047-4057. The identification of peptides from tumor tissue is described, for example, in Weinschenk et al., 2002, Integrated functional genomics approach for the design of patient-individual antitumor vaccines, CancerRes. 62:5818-5827.

In an additional preferred embodiment form, an additional step is performed according to step (h) in which the reactivity of leukocytes from the peripheral blood, preferably T-lymphocytes, against the peptides defined by step (h), is tested.

A further object is the method according to the invention, wherein the reactivity of peripheral leukocytes against the peptides defined by step (h) is tested by means of measuring γ-Interferon-mRNA and/or cytokin-mRNA synthesized by the leukocytes.

By detecting γ-Interferon-mRNA and/or cytokin-mRNA, it is possible to precisely prove the specific reactivity of leukocytes, preferably of T-lymphocytes against antigenic peptides. Both substances are secreted by activated T-lymphocytes after their activation by corresponding peptides which are bound to MHC-molecules on the cell surfaces. With this additional step, candidates of the already identified peptides can be identified even more precisely.

Yet another object is the method according to the invention, wherein, following step (h), a further step is performed, in which the presence of the specific T-lymphocytes is detected.

Using this method it is possible to specifically detect to what extent T-lymphocytes directed against isolated and identified peptides are pre-existing in patients. By performing this step it is possible to apply, as a vaccine, only those peptides for which T-lymphocytes are already pre-existing in the patient. The peptides can then be used to activate these specific T-lymphocytes.

A further object is the method according to the invention, wherein the detection of specific pre-existing T-lymphocytes is performed by labeling the leukocytes with reconstituted complexes of MHC molecules and antigenic peptide.

With this method the so-called tetramer-technology is utilized. A method for generating such reconstituted complexes ("tetramers") and for utilizing them is disclosed, for example, in Altman et al., 1996, Phenotypic analysis of antigen-specific T-lymphocytes, Science 274:94-96.

In an additional preferred method, specific T-lymphocytes from the peripheral blood of patients with reconstituted complexes made of MHC molecules and antigenic peptides, which are bound together with the molecule CD28 to a synthetic surface, are activated. This method is described, for example, in Walter et al., 2003, Cutting Edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres, J. Immunol. 171:4974-4979.

The invention involves, in an additional aspect, immunoreactive peptides which are identified and/or produced by the method according to the invention.

These peptides can be produced, after they are identified, in a targeted and specific way, i.e. synthesized chemically, in vitro or in vivo.

It is understood that in the process, at least one amino acid can be replaced by another amino acid with similar chemical properties, N- or C- terminal at least one additional amino acid can be present, at least one amino acid can be deleted, and/or at least one amino acid can be chemically modified, without the immunoreactive properties of the peptides going away.

Another object of the invention is a pharmaceutical composition comprising one or more peptides which have been identified and/or prepared by the method according to the invention.

The composition may be applied, for example, parenterally, for example subcutaneously, intradermally or intramuscularly. In the process, the peptides are dissolved or suspended in a pharmaceutical carrier; furthermore, the composition can contain excipients, such as for example, buffers, binders, etc. A comprehensive description of excipients, as they can be used in this type of composition, for example, is described in A. Kibbe, 2000, Handbook of Pharmaceutical Excipients, 3. Ed., American Pharmaceutical Association and Pharmaceutical Press. The peptides can also be administered together with immunostimulating substances, such as, for example, cytokines. A comprehensive description of immunostimulating substances, as they can be administered together with peptides, is given, for example, in Ribas et al., 2003, Current developments in cancer vaccines and cellular immunotherapy, J. Clin. Oncol. 21:2415-2432.

According one object of the invention the peptide may be used for treatment of tumor diseases and for preparing a medicament for treatment of tumor diseases.

Tumor diseases to be treated comprise renal, lung, intestinal, gastric, pancreas, breast, prostate, ovarial and/or skin cancer. This list of tumor diseases is only exemplary, and is not intended to limit the area of application.

The peptides can further be used for assessment of the therapy-course of a tumor disease.

Also, for other vaccines or therapies, peptides can be used to evaluate a course of treatment. Thus, the peptide according to the invention may not only be used for therapeutic, but also for diagnostic purposes.

In an additional embodiment form of the invention, peptides are used to produce antibodies.

Polyclonal antibodies may be obtained, in a conventional way, by the immunization of animals by means of injection of the peptides and the subsequent purification of the immunoglobulins from the blood of the immunized animals.

Monoclonal antibodies can be produced by standard protocols, such as described, for example, in Methods Enzymol., 1986, Hybridoma technology and monoclonal antibodies, 121:1-947.

Bispecific monoclonal antibodies can be produced according to standard protocols, such as, for example, Tomlinson et al., 2000, Methods for generating multivalentand bispecific antibody fragments, Methods Enzymol. 346:461-479.

In another aspect, the invention involves nucleic acid molecules which code for the peptide isolated by the method according to the invention.

The nucleic acid molecules can be DNA- or RNA-molecules and can be used for immune therapy of cancer as well.

According to one object of the invention the nucleic acid molecules can be provided in a vector.

A further object of the invention is a cell genetically modified by means of the nucleic acid molecule such that the cell produces a peptide identified according to the invention.

Another object of the invention is a method for preparing a tumor-associated peptide with which a peptide is identified according to the disclosed method and the identified peptide is synthesized chemically, *in vitro* or *in vivo*.

Peptides can be prepared by chemical reaction of amino acids, for example by the method of Merrifield, which is known in the art (see Merrifield, 1963, J. Am. Chem. Soc. 85:2149-2154).

Peptides can be produced in vitro, for example, in cell-free expression systems. Peptides can be manufactured in vivo in prokaryotic and eukaryotic cells.

A preferred embodiment form of the present invention is a method for producing a vaccine with the steps
(a) performing the disclosed method,
(b) producing the tumor-associated peptides identified in step (i)
(c) formulating the tumor-associated peptides produced in step (j)

The invention involves furthermore a diagnostic method in which the new method is performed and the presence and/or the quantitative ratio of a peptide as a diagnostic marker is used, a method for treating a pathological condition in which an immune response against a protein of interest is induced, whereby a therapeutically effective quantity of at least one of the peptides found according to the new method is administered, and an electronic storage medium that contains the amino acid sequence of at least one of the peptides according to the invention and/or the nucleic acid sequence of a nucleic acid molecule coding for a peptide according to the invention.

It is understood that aforementioned and subsequently to be explained characteristics can be applied not only in the respective combination given, but also can be used in other combinations or alone, without leaving the context of the invention presented here.

## Embodiment Examples

Embodiments of the invention are displayed and explained in the figures and the example below. Description of the drawings

**Fig. 1** an overview of the method according to the invention for identification and quantification of tumor-associated peptides;

**Fig. 2** the mass spectroscopic analysis of (A) non-modified and (B) $^{1}H_3/^{2}D_3$-acetylated peptides. (C) Mass spectroscopic analysis of a peptide mixture which, for example, contains both the non-modified peptide, the $^{1}H_3$-acetylated peptide and $^{2}D_3$-acetylated peptides with the amino acid sequence EVNGLISMY; (D) expains the nomenclature used in **Fig. 2**;

**Fig. 3** a comparative quantification of antigenic peptides from two different sources, where in (A) a mass-spectroscopic analysis is shown of the relative quantitative ratios of three different peptides made from two tissue samples (intestinal cancer sample, sample of normal tissue from the same patient). The peptides isolated from the intestinal cancer sample were $^{2}D_3$-acetylated. The peptides isolated from the normal tissue sample were $^{1}H_3$-acetylated. (B) shows a mass-spectroscopic analysis of three different peptides made of $^{1}H_4$-nicotinylated/guadinylated Awells cells and with keratin 18-transfected and $^{2}D_4$-nicotinylated / guadinylated Awells cells. (C) shows the determination of amino acid sequences of a $^{1}H_3$-acetylated peptide with the amino acid sequence DAAHPTNVQR and a $^{2}D_3$-acetylated peptide with the amino acid sequence DAAHPTNVQR by fragmentation;

**Fig. 4** Yields of peptides chemically modified in different ways. Four peptides with the amino acid sequences AETSYVKVL, KLSLGLPGL, SLGLQLAKV and VLDPRGIYL were added into a mixture in equimolar portions and then for the purpose of a comparative study of three procedural methods for chemical modification, they were either acetylated, or acetylated and guanidinylated, or guanidinylated and nicotinylated. After the conclusion of the chemical reaction for modification of the reference peptides, they were mixed with the non-modified peptides used at the beginning in order to then make a comparison possible in an analytical step. The comparative evaluation was performed by analysis using nano-electrospray ionization mass spectrometry (nano-ESI-MS).

## Experimental Procedures

### Patient Sample

From the Department for General Surgery of the University Clinic at the University of Tübingen, a sample was obtained from a patient who had been histologically confirmed to have intestinal cancer. The patient (referred to as CCA129 in the following) had the HLA class I type HLA-A01, HLA-A 68, HLA-B 08, HLA-B 44.

### Cell Line

The Awells cell line was used (European Collection of Cell Cultures, Porton Down, Salisbury, United Kingdom), which has the HLA Classe I Type HLA-A02, HLA-B-44.

### Keratin 18 Transfected Cell Line

The Awells cell line was stably transfected with the DNA sequence for human keratin 18 according to standard protocols. For this purpose, human cDNA coding Keratin 18 was subcloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany). The subsequent cloning was done between the restriction endonuclease interfaces EcoR I and Not I of the plasmid vector pcDNA3-li in the reading frame of the li-sequence. The transfection of the Awells cells was done using electroporation, and subsequently, stable transfectants were selected and kept in culture.

### Isolation of HLA class I-bound peptides

The preparation of the tissue sample which was shock frozen in liquid nitrogen after being surgically removed was performed as described by Schirle et al., Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach, 2000, European Journal of Immunology, 30:2216-2225. The peptides were isolated according to Standard Protocols, specifically using the monoclonal antibody W6/32, which is specific for the HLA Class I molecule. Barnstable et al., Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens - new tools for genetic analysis, 1978, Cell, 14:9-20, describes the production and use of this antibody.

### Acetylation of Peptides

10µl $^{1}H_6$-acetyl anhydride or $^{2}D_6$-acetyl anhydride (50% solution by volume in methanol) was added to 100µl peptide mixture (peptide quantities in mixtures: between 2 nmol and 200 pmol) in a 50%

methanol/water mixture (by volume). Th e chemical reaction occurred for 15 minutes at room temperature. The reaction was stopped by adding 1.1 µl formic acid. Then, the same volume was taken from both approaches and mixed together.

## Guadinylation of Peptides

Peptide mixtures from tumor tissue (CCA129), or Keratin 18 transfected or not-transfected Awells-cells (peptide quantities in mixtures: between 2 nmol and 200 pmol) in citrate buffer (50 mM citrate, pH 3.0) was mixed with 0.25 % trifluoroacetic acid (TFA, by volume), then the pH of the mixture was adjusted to 10.5 with 200 µl sodium hydroxide (10 M solution). After adding 1 ml O-methylisourea hemisulfate solution (2.5 M in water), the reaction mixture was incubated for 10 minutes at 65°C (water bath). The reaction was stopped by adding 200 µl formic acid.

## Nicotinylation of Guadinylated Peptides

The peptide mixtures, chemically modified by guadinylation, from tumor tissue (CCA129), or keratin-18-transfected or non-transfected Awells cells were put on a chromatography column of the type "reversed phase C-18 microcolumn" (AgilentTechnologies hydrophobic XGSXB) and washed with 0.5 ml water. Peptides bound to the column material were then left on the column and nicotinylated by slowly applying 1 ml of freshly prepared $^{1}H_4$- or $^{2}D_4$-nicotinyl-N-hydroxysuccini-mid-ester (sodium phosphate buffer 50 mM; pH 8.5) by chemical reaction at room temperature. Next, for a second time, 1 ml of freshly prepared $^{1}H_4$- or $^{2}D_4$-nicotinyl-N-hydroxysuccinimide ester was slowly conducted through the chromatography column loaded with the peptide mixture. After that, hydroxylamine is guided through the column in order to again remove undesired modifications of tyrosin residues by nicotinyl groups. Then, the chromatography column is washed with water before the peptides are eluted from the column with 100 µl of a 50% acetonitrile/water mixture (by volume).

## Offline-High Performance Liquid Chromatography (HPLC) Separation of peptide mixtures

Mixtures of peptides chemically modified in this way were mixed in equimolar ratios und contracted in volume to approx. 100 µl by vacuum centrifugation. The contracted mixtures were diluted with 400 µl of water with 0.08 % TFA (by volume), before they were put by automated sample injection onto a "reversed phase"-chromatography column, model µRP SC C2/C18, 100 mm x 2.1 mm (Amersham-Pharmacia, Freiburg, Germany) which was connected to a SMART-HPLC-System (Amersham-Pharmacia, Freiburg, Germany). For chromatographic separation and the elution of the peptides bound to the column material, a binary gradient made from two solvent mixtures A and B was used. Solvent mixture A contains 0.1 % TFA (by volume) in water. Solvent mixture B contains 0.08 % TFA and 80 % acetonitrile (both by volume) in water. The binary gradient begins with 90 % solvent mixture A and 10 % solvent mixture B and follows a linear progression until a mixture ratio of 40 % solvent mixture A and 60 % solvent mixture B. The eluate is collected in fractions with a volume of 150 µl per fraction. Prior to beginning the mass spectrometric studies of the chromatographically separated peptides, the collected fractions are completely dried by vacuum centrifugation and then dissolved again in a mixture of 50 % methanol, 49.9 % water and 0.1 % formic acid.

## Microcapillary Liquid Chromatrography Mass Spectrometry

The peptide mixtures were analyzed using a reversed-phase-HPLC-Systems ("reversed phase Ultimate HPLC System, Dionex, Amsterdam, Niederlande) connected to a hybrid quadropole mass spectrometry device ("orthogonal acceleration time of flight mass spectrometer", Micromass, Manchester, United Kingdom) equipped with a micro-electrospray ionisation source. For this purpose, the sample material was first desalinated and preconcentrated on a C18-precolumn with the dimensions 300 µm x 5 mm (LC Packings, Amsterdam, Netherlands). A syringe pump (Harvard Apparatus, Inc.) equipped with a gastight 100 µl-syringe (1710 RNR, Hamilton) delivered solvent and sample at a rate of 2 µl per minute. The precolumn loaded with the peptide mixture is then connected in the flow direction in front of a silica column (75 µm x 250 mm, Dionex, Amsterdam, Netherlands) that is connected to a "reversed phase Ultimate"-HPLC-System and loaded with C18-reversed-phase material (5 µm, Dionex, Amsterdam, Netherlands). For the elution of the bound

peptides, a binary gradient is planned over a time period of 120 minutes, which begins with 15 % solvent A (4 mM ammonium acetate in water, pH 3.0) and 85 % solvent B (2 mM ammonium acetate in a mixture by volume from 80 % acetonitrile and 20 % water, pH 3.0) and leads to a mixture ratio of 40 % solvent A and 60 % solvent B. The flow-through rate during the elution of the peptides is reduced by the Ultimate split-System (Dionex, Amsterdam, Netherlands) to ca. 300 µl per minute. The eluate was introduced into the micro-ESI-source through a gold-coated glass capillary (PicoTip, New Objective, Cambridge/Massachusetts, U.S.A.). The integration time for the "time of flight" analysis (TOF analyzer) was set to 1 second, the delay time between two analysis operations was 1/10 second. The ratio of chemically modified peptides with deuterium (2D) atoms to peptides having the same basic amino acid sequence with normal hydrogen (1H) atoms was determined by comparing the relative height of the "peaks" (measured vertex points of the predominate signal from the mass spectrometric analysis).

The online fragmentation of peptides to determine the amino acid sequence (HPLC-MSMS) was performed with an integration time of 4 seconds for the "time of flight" analysis (TOF analyzer) and a delay time of 1/10 second between two analysis operations, and otherwise as described. In the process of the online fragmentation of the $[M + H]^+$ and $[M + H]^{2+}$ ions, a change is made automatically between the HPLC-MS mode and HPLC-MSMS mode. The spectra that emerged from the mass spectrometric analyses were analyzed manually. Data base searches (NCBInr, EST) were performed using MASCOT (http://www.matrixscience.com).

In an additional preferred embodiment for small sample volumes, instead of an HPLC system, metal-coated glass capillaries (Proxeon, Odense, Denmark) could also be used for further reduction of the flow rate in bringing the sample to the micro-ESI-source. In this way, flow rates of 20 nl per minute up to 50 nl per minute are possible. In this embodiment form, the ratio of chemically modified peptides with deuterium ($^2$D) atoms to peptides having the same basic amino acid sequence with normal hydrogen ($^1$H) atoms was determined by comparing the relative height of the "peaks" (measured vertex points of the predominate signals from the mass spectrometric analysis) and the relative mathematically integrated areas of the "peaks". The fragmentation of peptides in HPLC-MSMS mode is also possible in this embodiment form. This is performed with collision energies of 30-60 eV for $[M + H]^+$-ionized fragments and 20-30 eV for $[M + H]^{2+}$-ionized fragments. For this embodiment form, the integration time for the "time of flight" analysis (TOF analyzer) is 1 second, and between two analysis operations, there is a delay time of 1/10 of a second.

## Results

**Fig. 1** depicts the basic principle for differential measurement and identification of MHC-class I bound peptides. In this method, peptides from two different sources are treated with reactive chemical groups which can be distinguished by the presence or absence of certain hydrogen species (light hydrogen: $^1$H; heavy hydrogen: $^2$D), without the physical properties, which are achieved by the different hydrogen isotopes and which are used for differentiation, having a measurable influence on the chemical properties of the modified peptides. The peptide derivatives occurring as a result of the chemical modification are combined with each other, i.e. mixed and/or amalgamated, and separated by chromatography ("offline"-HPLC or "online"-HPLC-MS) according to their hydrophobicity, or respective hydrophilicity, based on the primary amino acid sequence. The signal intensity of the specific mass/charge signals determined by the subsequent mass spectrometric analysis is the indicator for the relative quantitative ratio between peptides which have the same basic primary amino acid sequence and which were obtained from different sources. The use of the tandem-MSMS method provides additional information, using databases, about the amino acid sequence of the peptides present in the individual case.

The acetylation of MHC class I ligands represents a fast and simple method for the chemical modification of peptides. The acetylation of peptides was optimized experimentally using synthetic peptide mixtures. After a 15 minute reaction time (as previously described), the peptides were acetylated completely on the amino-terminal end.

The use of $^2D_6$- and $^1H_6$-acetanhydride for the acetylation makes possible the differential quantification of peptides.

In order to demonstrate feasibility, MHC class I bound peptides were obtained from MGAR cells as described above, separated into two equal-volume partial samples and acetylated as described with $^2D_6$-acetanhydride, or respectively $^1H_6$-acetanhydride. After the end of the chemical reaction, the partial samples were again mixed in equimolar ratios and the relative ratios between $^2D_3$- and $^1H_3$-acetylated peptides were determined. **Fig. 2** shows, for example, the $^2D_3$- and $^1H_3$-variants of a peptide with the amino acid sequence EVNGLISMY (molecular weight without chemical modification: 1040.5 Da). The peptide EVNGLISMY constitutes a fragment from the "U5 snRNP-specific Protein". The measured relative ratio between $^2D_3$- and $^1H_3$-variants ($^2D_3/^1H_3$-ratio) of EVNGLISMY was 1.0. For 15 additional peptides, which had been eluted from the same MGAR cells and had been detected by mass spectrometry as singly or doubly charged ions, the $^2D_3/^1H_3$-ratio had an arithmetic mean of 1.01. The standard deviation (SD) was ±0.13 (Table 1). In addition to making possible a determination of the relative portions of peptides with the same basic amino acid sequence from two or more different sources, the acetylation of peptides by the shifting of the b-series-ions by 3 Da of $^2D_3$-acetylated relative to $^1H_3$-acetylated peptides also leads to a simplification of the valuation of the corresponding mass spectrogram **(Fig.** 2C). b-series ions are generally ionized fragments of both chemically modified as well as non-modified peptides which contain at least the amino acid residue placed at the amino terminus in the amino acid primary sequence. y-series ions are, as opposed to b-series ions, generally ionized fragments of both chemically modified, as well as non-modified peptides, which contain at least the amino acid residue placed at the carboxyl terminus in the amino acid primary sequence.

It is disadvantageous in the acetylation of peptides that the ionization by the introduction of the acetyl residue onto the amino-terminus end of the peptides can cause a positive charge less than in a peptide with an intact N-terminus. Since in principle, multiply charged peptides can be better detected by mass spectrometry than singly charged peptides, the acetylation also causes a loss of sensitivity. The experiments performed also showed that acetylation of the ε-amino group of lysine residues can occur. This acetylation of the ε-amino group of lysine residues also has the result that by the ionization, a positive charge less than in the peptide not chemically modified by acetylation can occur. The resultant loss in sensitivity, however, applies to the same extent for the basic peptides of the same amino acid primary sequence from the differently used sources, so that a measurable effect on the inner ratio between the associated signals of the equivalently sequenced, in one case $^2D_3$-acetylated peptides and in the other case $^1H_3$-acetylated peptides, does not occur.

Identification of MHC class I-bound peptides from tissue samples of intestinal tumors and tissue samples of normal tissue surrounding the tumors using $^2D_3$- and $^1H_3$-acetylation of the MHC class I-bound peptides in a mass spectrometric analysis comparing the relative quantities based on electrospray ionization mass spectrometry (ESI-MS).

Peptides were isolated as described from MHC classe I molecules of an intestinal cancer sample (CCA129) and from MHC class I molecules of a sample of the normal tissue surrounding the surgically removed tumor and then chemically modified by $^2D_3$- (tumor) and $^1H_3$-(normal tissue) acetylation. After the chromatographic separation of the modified peptides by microbore-HPLC, 19 peptides were identified as described by nano-ESI-MS. For 17 of these 19 peptides, the relative quantitative ratio could be determined by a comparison of the specific peptides of the tumor tissue sample to the peptides of the sample of normal tissue with the same basic amino acid sequence. The majority of the identified peptides was present in similar quantities in both the samples examined ($^2D_3/^1H_3$ ratios between 1.07 and 2.42). On the whole, a 1.7 times greater quantity of peptides was present in the tumor sample compared to the normal tissue sample. Two peptides were overrepresented in the tumor tissue, and one peptide was underrepresented in the tumor. The statistical evaluation of the results using the "student's t-test" confirmed that only the two over-expressed and the one under-expressed peptide was outside a 99.99% confidence intervall of 0.87 to 2.56.

The two peptides over-represented in the tumor derived from the human proteins "ribosomal protein L24" and beta-catenin. While little data with regard to a tumor-association exists for the ribosomal protein L24, a role in the origination of esophageal cancer was described for the "ribosomal protein L15", which is related to the "ribosomal protein L24", by Wang et al., 2001, Cloning and characterization of full-length human ribosomal protein L15cDNA which was overexpressed in

esophageal cancer, Gene 263:205-209. For beta-catenin, on the other hand, a function as an oncogen, which turns on by transactivation of other oncogens, such as, for example, the matrix metalloproteinase MMP-7, was described by Ougolkov et al., 2002, Oncogenic beta-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer, Gastroenterology 122: 60-71. A mutated beta-catenin peptide was described by Robbins et al., 1996, A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor-infiltrating lymphocytes, Journal of Experimental Medicine 183:1185-1192, as a target structure in connection with the human MHC-Allel HLA-A-24 for CD8-positive, skin cancer-infiltrating T cells.

Improved yield of MHC classe I-bound peptides after chemical modification of the peptides by O-methylisourea-hemisulfate and nicotinyl-N-hydroxy-succinimide ester (NicNHS).

The initial and new combination of two methods for chemical modification of peptides by combination of the einheitlichen guadinylation of ε-amino groups of lysin residues in peptides by O-methylisourea-hemisulfate and the nicotinylation of α-amino groups of peptides by NicNHS leads to a clear improvement of the ionization of peptides (**Fig. 4**). In order to simplify the desalination of the chemically modified peptides, the nicotinylation of the peptides is performed on a C18-chromatography column as described above. T he undesired modification of the side-chains of tyrosine residues induced by the nicotinylation could be removed again by treatment of the modified peptides with hydroxylamine. For example, it is shown using the peptide with the amino acid sequence AETSYVKL in **Fig. 4**, that the nicotinylation of the N-terminus affects the ionization in such a way that the nicotinylated peptides can be detected as well as the peptides that have not been chemically modified.

Identification and Quantification of MHC class I bound peptides from the Awells cell line and the Awells cell line transfected with a plasmid containing the cDNA of the human keratin 18 by guadinylation and $^{2}D_{4}$-/$^{1}H_{4}$-nicotinylation of the peptides.

In Trask et al., 1990, Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells, Proc. Natl. Acad. Sei. U.S.A., 87:2319-2323, it was shown that keratines are suitable as markers for distinguishing tumor and normal tissue. In order to identify new MHC classe I-bound peptides from human keratin 18 and to demonstrate the differential quantification using an exemplary tumor antigen, peptides were isolated from the non-transfected (Awells) and from the Awells cell line transfected with the named plasmid (Awells keratin 18). The isolated peptide mixtures were then chemically modified as described by guadinylation and $^{2}D_{3}$-, respectively $^{1}H_{3}$-nicotinylation. Th e chemically modified peptide mixtures were mixed together and examined by HPLC-MS analysis as described. In a second experiment, work was done in MSMS mode, so that the amino acid sequences of in total 27 different peptides could be determined. All 27 peptides found, with the exception of a peptide with a molecular weight of 1091.6 Da were both transfected as well as detected on non-transfected cells in quantities which were within the confidence interval of 0.64 to 2.28 (statistical evaluation using the "Student's t-test"). For the peptide with a molecular weight of 1091.6 Da, the amino acid sequence RLASYLDRV was determined using MSMS analysis, which is a fragment of the amino acid sequence of keratin 18. The MSMS spectra, which led to identification of the peptide with the sequence RLASYLDRV, are shown in **Fig.** 3D. For the peptide RLASYLDRV, no signal could be detected which could be linked to a chemical modification of the primary sequence with a $^{1}H_{3}$-nicotinyl residue. This observation suggests that keratin 18 was expressed exclusively in the Awells keratin 18 cells. The signal for the peptide RLASYLDRV with a $^{2}D_{3}$-nicotinyl residue, on the other hand, was expressed six times greater than the background.

The described method of guadinylation and nicotinylation of peptides using the two hydrogen isotopes $^{1}H$ and $^{2}D$ allows for the first time the fast and exact determination of relative quantitative differences between sequence-equivalent peptides from two or more different sources. By the use of the method for guadinylation and nicotinylation of peptides on samples of tumor tissue and normal tissue of the same organ, or by the use of the mentioned method on cell lines which had been transfected before by nucleic acids coding for oncogenes or other tumor-associated gene products, tumor-associated peptide antigens can be determined which are especially suitable for the production of vaccines for cancer treatment.

In Tables 1 to 3, the results described above are compiled. Table 4 shows the allocation of the peptides found to the source protein and gives its associated SEQ ID no. from the sequence protocol. The sequence protocol shows the peptides according to the invention.

Table 1 shows the measured values of mass spectrometric signal intensities from an equimolar mixture of $^2D_3$-nicotinylated peptides to $^1H_3$-nicotinylated peptides from MGAR-cells and the derived ratios of $^2D_3$-nicotinylated peptides to $^1H_3$-nicotinylated peptides each with the respective same basic amino acid sequences. Peptides with double positive charge $[M + H]^{2+}$ have a dramatically higher signal intensity than peptides with a single positive charge $[M + H]^+$. Peptide ions with double positive charge $[M + H]^{2+}$ have different basic sequences than peptides with a single positive charge $[M + H]^+$.

| **Peptide Ionization condition** | **m/z-ratio $^1H_3$-acetylated peptide ions** | **$^1H_3$ peptide signals [counted ions]** | **$^2D_3$- peptide signals [counted ions]** | **$^2D_3/^1H_3$-Ratio** |
|---|---|---|---|---|
| | 554.45 | 409 | 361 | 0.88 |
| | 567.99 | 590 | 511 | 0.86 |
| | 574.50 | 316 | 306 | 0.96 |
| $[M+2H]^{2+}$ | 578.52 | 884 | 877 | 0.99 |
| | 582.01 | 615 | 598 | 0.97 |
| | 597.96 | 612 | 800 | 1.30 |
| | 604.95 | 499 | 558 | 1.11 |
| | 612.49 | 515 | 502 | 0.97 |
| | 941.49 | 33 | 32 | 0.97 |
| | 992.51 | 39 | 51 | 1.30 |
| | 1028.55 | 23 | 20 | 0.87 |
| | 1039.47 | 21 | 22 | 1.04 |
| $[M+H]^+$ | 1057.56 | 30 | 32 | 1.06 |
| | 1083.48 | 63 | 63 | 1.00 |
| | 1092.47 | 59 | 54 | 0.92 |
| | 1170.47 | 77 | 70 | 0.90 |
| | **Mean value** | | | **1.01** |
| | **Standard deviation** | | | **±0.13** |

Table 2 shows sequences and measurement results for natural peptides obtained from HLA class I molecules from tumor tissue and normal tissue of a patient with intestinal cancer. To perform the analysis, the peptides isolated from the tissue samples were chemically modified with $^{2}D_{6}$-acetic anhydride (tumor tissue) or $^{1}H_{6}$-acetic anhydride (normal tissue), mixed, identified according to the method by mass spectrometric analysis, and the quantitative ratios occurring between the tissue samples, of peptides with identical amino acid sequences were quantified by determining the $^{2}D_{3}/^{1}H_{3}$ ratio. In order to specify significant over-representation and/or under-representation of identified and quantified peptides, the measurement results ($^{2}D_{3}/^{1}H_{3}$ ratio) were evaluated by statistical analysis (Student's t-test).

| **Peptide Sequence (HLA anchor residues in bold)** | **HLA** | **Protein source** | **AA-Position** | **Ratio $^{2}D_{3}/^{1}H_{3}$** |
|---|---|---|---|---|
| T T E Q H G **AR** **Y** | **A*01** | Tapasin | **372-380** | **n.d.** |
| F T **K** **VK** P L **L** | **B*08** | Myosin heavy chain, nonmuscle | **831-838** | **n.d.** |
| **VA** V G V A R A **R** | **A*68** | Poly IG receptor | **656-664** | **0.69** |
| D V S H T V V L **R** | **A*68** | Translocon-associated protein ß- | **88-96** | **1.07** |
| T **L** G D I V F K **R** | **A*68** | Fatty acid-bindin protein, liver | **114-122** | **1.14** |
| D I **H** **HK** V L S **L** | **B*08** | Ras-GAP SH3 binding protein 2 | **60-68** | **1.30** |
| E **VT** R I L D G **K** | **A*68** | SH3BGR3-like protein | **23-31** | **1.32** |
| R **V** A P E E H P V **L** | **n.a.** | Actin, cytoplasmic 1 | **94-103** | **1.39** |
| T **T** A E R E I V **R** | **A*68** | Actin alpha | **204-212** | **1.45** |
| S **I** F D G R V V A **K** | **A*68** | Putative membrane protein | **88-97** | **1.45** |
| E **A** G P S I V H **R** | **A*68** | Actin alpha | **366-374** | **1.51** |
| **DT** A A Q I T Q **R** | **A*68** | MHC class I antigen (HLA-B) | **136-144** | **1.61** |
| **DT** I E I I T D **R** | **A*68** | HNRPA2/BI | **139-147** | **1.66** |
| E **S** T G S I A K **R** | **A*68** | AldolaseA | **34-42** | **1.73** |
| A **VA** A V A A R **R** | **A*68** | Glucosidase II alpha subunit | **3-11** | **1.91** |
| T **A** A D T A A Q I T **R** | **A*68** | MHC class I antigen (HLA-B) | **133-144** | **2.19** |
| E **S** G **P** S I V H **R** | **A*68** | Actin beta | **364-372** | **2.42** |
| **DA** A H P T N V Q **R** | **A*68** | Beta-catenin | **115-124** | **3.02** |
| S **L** A D I M A K **R** | **A*68** | Ribosomal protein L24 | **86-94** | **3.27** |
| **Mean value** | | | | **1.71** |
| **Standard deviation** | | | | **±0.68** |
| **Student-t test:** Confidence interval 99.99 % (n= 27) | | | | **0.87-2.56** |

n.a., not assigned; n.d., not determined

Table 3 shows sequences and measurement results for natural peptides obtained by HLA-class I molecules from the unchanged Awells cell line and the Awells cell line genetically altered as described by transfection with keratin 18. From the two sources named, Awells and Awells keratin 18, isolated peptides were changed by chemical modification with $^{1}H_4$-nicotinyl residues (Awells) or $^{2}D_4$-nicotinyl residues (Awells keratin 18). According to the method, peptides were mixed after the end of the chemical modification and identified by mass spectrometric analysis and the quantitative ratios existing between Awells and Awells keratin 18 of peptides with identical amino acid sequences was quantified by determining the $^{2}D_4/^{1}H_4$ ratio. In order to specify significant over-representation and/or under-representation of identified and quantified peptides, the measurement results ($^{2}D_3/^{1}H_3$ ratio) were evaluated by statistical analysis (Student's t-test).

| **Peptide Sequence (HLA anchor residues in bold)** | **HLA** | **Source protein** | **AA-Position** | **Ratio ($^{2}D_4/^{1}H_4$)** |
|---|---|---|---|---|
| KE S T L H L V **L** | B*44 | Ubiquitin | 63-71 | 0.96 |
| A **E** S L L T M E **Y** | B*44 | KIAA1390 | 31-39 | 1.02 |
| L **L** M E H T M V A **F** | A*02 | EST | 24-33 | 1.05 |
| HL A V E R G K **V** | A*02 | Similartox-kinase | 532-540 | 1.06 |
| S **E** I E A K VR **Y** | B*44 | Talin I | 290-298 | 1.08 |
| T **L** F P G K V H S **L** | A*02 | WD-repeat protein 6 | 432-441 | 1.09 |
| S **E** D N R I L L **W** | B*44 | Methylosome protein 50 | 187-195 | 1.10 |
| S **I** I G R L L E **V** | A*02 | PhosphatasePPl-al | 11-19 | 1.12 |
| YL L P A I V H **I** | A*02 | RNA-dependent helicase p68 | 148-156 | 1.12 |
| **Q** L VD I I E K **V** | A*02 | Proteasome activator complex SU 3 | 114-122 | 1.14 |
| AL L D K L Y A **L** | A*02 | Simiiarto mitochon. ribosom. prot. | 78-86 | 1.14 |
| I **E** H G I I T N **W** | B*44 | Actin alpha skeletal muscle | 73-81 | 1.19 |
| I **M** L E A L E R **V** | A*02 | Small nuclear ribonucleoprotein G | 68-76 | 1.19 |
| L **L** F D R P M H **V** | A*02 | HNRP M | 268-276 | 1.22 |
| AE K L I T Q T **F** | B*44 | NPDOM | 2-10 | 1.23 |
| R **L** A Q H I T Y **V** | A*02 | Licensing factor MCM7 | 532-540 | 1.25 |
| S **E** P D F V A K **F** | B*44 | FLJ2067I | 121-129 | 1.28 |
| T **E** V T G H R **W** | B*44 | Basigin | 48-55 | 1.37 |
| AE T P D I K L **F** | B*44 | 40S ribosomal protein S5 | 12-20 | 1.48 |
| QE H VK S F S **W** | B*44 | Sortilin-related receptor | 245-253 | 1.50 |
| A*I* V D K V P S **V** | A*02 | Coatomer gamma subunit | 147-155 | 1.52 |
| E **E** P T V I K K **Y** | B*44 | Sorting nexin 5 | 257-265 | 1.54 |
| QE A G I K T A **F** | B*44 | Multifunctional protein ADE2 | 69-77 | 1.60 |
| GE A S L R L A H **Y** | B*44 | HistoneH2B.f | 75-83 | 1.65 |
| QE D L R T F S **W** | B*44 | Ras-GTPase-activating protein | 243-251 | 1.66 |
| M **E** Q V I F K Y **L** | B*44 | Actin-like protein 3 | 93-101 | 1.98 |
| R **L** A S Y L D R **V** | A*02 | Cytokeratin 18 | 89-97 | ≥5.95 |
| **Mean value** | | | | **1.46** |
| **Standard deviation** | | | | **±0.93** |
| **Student-t test:** Confidence interval 99.99 % (n=27) | | | | **0.64-2.28** |

Table 4 shows the sequences, the source protein and/or gene, the relative position of the peptide within the source protein by indicating the amino acid positions, the gene bank accession numbers and the SEQ ID no. associated with the respective sequences:

| | Sequence | Position/Genart | Acc. No. | Seq. ID-No. |
|---|---|---|---|---|
| 1. | TTEQHGARY | 372-380 Tapasin | NP_003181 | SEQ ID-Nr. 1 |
| 2. | FTKVKPLL | 831-838<br>Myosin heavy chain, noranuscle A | NP_0024 64 | SEQ ID-Nr. 2 |
| 3. | VAVGVARAR | 656-664<br>Poly IG receptor | NP_002635 | SEQ ID-Nr. 3 |
| 4. | DVSHTVVLR | 88-96 (NVSHTVVLR deamidiert)<br>Translocon-associated protein, beta subunit | NP_003136 | SEQ ID-Nr. 4 |
| 5. | TLGDIVFKR | 114-122<br>Fatty acid-blnding protein, liver | NP 001434 | SEQ ID-Nr. 5 |
| 6. | DIHHKVLSL | 60-68<br>Ras-GAP SH3 binding protein 2 | NP_036429 | SEQ ID-Nr. 6 |
| 7. | EVTRILDGK | 23-31<br>SH3BGR3-like protein | NP_112576 | SEQ ID-Nr. 7 |
| 8. | RVAPEEHPVL | 95-104<br>Actin, cytoplasmic 1 | NP_001092 | SEQ ID-Nr. 8 |
| 9. | EAGPSIVHR | 366-374<br>Actin alpha | NP_005150 | SEQ ID-Nr. 9 |
| 10. | ESTGSIAKR | 35-43<br>Aldolase A | NP_00025 | SEQ ID-Nr. 10 |
| 11. | TAADTAAQITR | 133-143<br>MHC class I antigen (HLA-B) | BAA84116 | SEQ ID-Nr. 11 |
| 12. | DAAHPTNVQR | 115-124<br>Beta-catenin | NP_001895 | SEQ ID-Nr. 12 |
| 13. | AESLLTMEY | 31-39<br>KIAA1390 | BAA92628 | SEQ ID-Nr. 13 |
| 14. | LLMEHTMVAF | 470-461<br>EST (rf-1) | BG752065 | SEQ ID-Nr. 14 |
| 15. | HLAVERGKV | 532-540<br>x-kinase | AAM91924 | SEQ ID-Nr. 15 |
| 16. | SEIEAKVRY | 290-298<br>Talin 1 | NP_006280 | SEQ ID-Nr. 16 |

| | | | | |
|---|---|---|---|---|
| 17. | TLFPGKVHSL | 432-441<br>WD-repeat protein 6 | NP_060501 | SEQ ID-Nr. 17 |
| 18. | SEDNRILLW | 187-195<br>Methylosome protein 50 | NP_077007 | SEQ ID-Nr. 18 |
| 19. | SIIGRLLEV | 11-19<br>Serine/threonine protein phosphatase PPl-alpha 1 | NP_002699 | SEQ ID-Nr. 19 |
| 20. | QLVDIIEKV | 114-122<br>Proteasome activator complex subunit 3 | NP_005780 | SEQ ID-Nr. 20 |
| 21. | ALLDKLYAL | 78-86<br>Similar to mitochondrial ribosomal protein S4 | NP_060755 | SEQ ID-Nr. 21 |
| 22. | IMLEALERV | 68-76<br>Small nuclear ribonucleoprotein G | NP_003087 | SEQ ID-Nr. 22 |
| 23. | AEKLITQTF | 2-10<br>NPD011 | AAG44476 | SEQ ID-Nr. 23 |
| 24. | RLAQHITYV | 532-540<br>DNA replication licensing factor MCM7 | NP_005907 | SEQ ID-Nr. 24 |
| 25. | SEPDFVAKF | 121-129 FLJ20671 | NP_060394 | SEQ ID-Nr. 25 |
| 26. | TEVTGHRW | 164-171 Basigin | NP_001719 | SEQ ID-Nr. 26 |
| 27. | AETPDIKLF | 12-20<br>40S ribosomal protein S5 | NP_001000 | SEQ ID-Nr. 27 |
| 28. | QEHVKSFSW | 245-253<br>Sortilin-related receptor | NP_003096 | SEQ ID-Nr. 28 |
| 29. | EEPTVIKKY | 257-265 Sorting nexin 5 | NP_055241 | SEQ ID-Nr. 29 |
| 30. | QEAGIKTAF | 69-77<br>Multifunctional protein ADE2 | NP_006443 | SEQ ID-Nr. 30 |
| 31. | GEASRLAHY | 76-84 Histone 2B.f | NP_066406 | SEQ ID-Nr. 31 |
| 32. | QEDLRTFSW | 243-251 Ras-GTPase-activating protein | NP_005745 | SEQ ID-Nr. 32 |
| 33. | MEQVIFKYL | 93-101<br>Actin-like protein 3 | NP_005712 | SEQ ID-Nr. 33 |
| 34. | RLASYLDRV | 90-98<br>Cytokeratin 18 | NP_000215 | SEQ ID-Nr. 34 |

| | | | | |
|---|---|---|---|---|
| 35. | IEHGIITNW | 73-81<br>Actin,alpha skeletal muscle | NP_001091 | SEQ ID-Nr. 35 |
| 36. | TTAEREIVR | 204-212<br>Actin, alpha skeletal muscle | NP_001091 | SEQ ID-Nr. 36 |

Sequence listing

<110> Immatics Biotechnologies GmbH

<120> Method for identification and quantification of tumor-associated peptides

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**Patent Claims**

1. Method for identification and quantification of tumor-associated peptides, with the steps: Preparation of a first sample of tissue or cells,
Preparation of a second sample of tissue or cells with the same weight quantity or cell number as the first sample,
Extracting peptides from the first and the second sample,
separate, chemically identical modification of peptides from both samples to generate different physical properties in the peptides from the different samples, mixing the peptides modified in this way from two samples, determining the amino acid sequences of the peptides, and determining the relative quantitative ratios of sequence-identical peptides from the two samples using the different physical properties.

2. Method according to claim 1, characterized in that the peptides from the two samples are chemically modified using at least two different stable isotopes of the same element.

3. Method according to claim 1 or 2, characterized by the following steps:

a) Preparing a sample from tumorous tissue and a sample from corresponding normal tissue or correspondingly transfected and/or infected cell lines, whereby both samples have the same quantities by weight or cell numbers,
b) Isolation of peptides from the sample made from tumorous tissue,
c) Isolation of peptides from the sample made from the corresponding normal tissue,
d) Chemical change of the peptides obtained from step (b) with a chemical group that contains a stable isotope of an element from the Periodic Table of the Elements,
e) Chemical change of the peptides obtained from step (c) with a chemical group that contains a second stable isotope of the element used in step d) from the Periodic Table of the Elements, whereby the stable isotope used in this step is lighter or heavier than the isotope used in step d),
f) Mixing of the chemically modified peptides obtained from steps (d) and (e),
g) Separation of the peptides obtained from step f) by a chromatographic process,
h) Identification and determining of peptides with identical amino acid sequences and the quantitative ratios of the chemically modified peptides with identical amino acid sequences from step (g), i) Identification of tumor-associated peptides suitable for the composition of a therapeutic vaccine based on the data obtained from step (h).

4. Method according to one of the claims 1 to 3, characterized in that the tumor-associated peptides are MHC class I ligands.

5. Method according to claim 3 or 4, characterized in that in step d) deuterium ($^2D$) and in step e) normal hydrogen ($^1H$) are used as stable isotopes.

6. Method according to one of the claims 3 to 5, characterized in that in step g) a chromatographic separation of the peptides by HPLC is performed.

7. Method according to one of the claims 3 to 6, characterized in that the step h) is done by mass spectrometric analysis.

8. Method according to claim 7, characterized in that the mass spectrometric analysis for determining the quantitative ratios of two peptides with identical amino acid sequence and identical chemical modification is performed using at least two different isotopes of the same element from the Periodic Table of the Elements.

9. Method according to claim 8, characterized in that the relative intensity of measured signals for peptides of identical amino acid sequences and identical chemical modification with simultaneously present different isotopes of the same element serves to calculate the relative quantitative ratios between the named peptides.

10. Method according to one of the claims 3 to 9, characterized in that in step i) suitable databases for identifying tumor-associated genes and gene products are used.

11. Method according to claim 3 to 10, characterized in that after step h) a further step is performed, in which the reactivity of peripheral leukocytes, preferably T-lymphocytes, is tested against the identified and quantified peptides.

12. Method according to claim 11, characterized in that the reactivity test is performed by means of measuring cytokine-mRNA and/or interferon mRNA synthesized by the leukocytes.

13. Method according to claim 11, characterized in that the test of the reactivity is done by the activation of peripheral T-lymphocytes by means of reconstituted complexes made from molecules and peptides presenting antigens.

14. Method according to claim 13, characterized in that the complexes used for activating T-lymphocytes from antigen-presenting molecules and peptides are fixed on a surface.

15. Method according to claim 14, characterized in that the surface used for fixing the complexes made from the molecules and peptides presenting antigens consists of polystyrene.

16. Method according to one of the claims 13 to 15, characterized in that the molecules presenting antigens are connected to biotin by a chemical reaction and the surface used is made of polystyrene coated by chemical reaction with streptavidin.

17. Method for producing a peptide, in which according to the method according to one of the claims 1 to 16, a peptide is identified and the identified peptide is synthesized chemically, in vitro, or in vivo.

18. Peptide which was identified by a method according to one of the claims 1 to 16 and/or produced by a method according to claim 17.

19. Tumor-associated peptide with an amino acid sequence chosen from the group consisting of SEQ-ID no. 1 to 36 from the attached sequence protocol, whereby the peptide is capable of binding to a molecule of the human major histocompatibility complex (MHC) class I.

20. Peptide according to claim 18 or 19, characterized in that at least one amino acid is replaced by another amino acid with similar chemical properties.

21. Peptide according to one of the claims 18 to 20, characterized in that N-terminus or C-terminus at least one additional amino acid is present.

22. Peptide according to one of the claims 18 to 21 characterized in that at least one amino acid is deleted.

23. Peptide according to one of the claims 18 to 22 characterized in that at least one amino acid is chemically modified.

24. Use of one or more of the peptides according to one of the claims 18 to 23 for producing a drug for treatment of tumor diseases and/or adenomatous diseases.

25. Use of the peptide according to one of the claims 18 to 23 for treatment of tumor diseases and/or adenomatous diseases.

26. Use according to claim 24 or 25, characterized in that the disease is renal, lung, intestinal, gastric, pancreatic, breast, prostate, ovarial and/or skin cancer.

27. Use according to one of the claims 24 to 26, characterized in that the peptide is used together with an adjuvant.

28. Use according to one of the claims 24 to 27, characterized in that a peptide bound to a cell presenting an antigen is used.

29. Use of the peptide according to one of the claims 18 to 23 for marking leukocytes, in particular T-lymphocytes.

30. Use of the peptide according to one of the claims 18 to 23 to evaluate a therapy progression for a tumor disease.

31. Use of a peptide according to one of the claims 18 to 23 to produce an antibody.

32. Pharmaceutical composition containing one or more of the peptides according to one of the claims 18 to 23.

33. Nucleic acid molecule coding for the peptide according to one of the claims 18 to 23.

34. Vector, containing the nucleic acid molecule according to claim 33.

35. Cell which, using the nucleic acid molecule according to claim 33, or with the vector according to claim 34, was genetically changed in such a way that it expresses a peptide according to one of the claims 18 to 23.

36. Use of the nucleic acid molecule from claim 33 and/or the vector from claim 34 and/or the cell from claim 35 to manufacture a drug for treatment of tumor diseases and/or adenomatous diseases.

37. Method for producing a vaccine with the steps:
a) Performing the method according to one of the claims 1 to 16,
b) Producing the identified peptides and formulating the peptides produced into the vaccine.

38. Diagnostic method in which the method according to one of the claims 1 to 16 was performed and the presence and/or the quantitative ratio of a peptide is used as a diagnostic marker.

39. Method for treating a pathological condition, in which an immune response is induced against a protein that is of interest, characterized in that a therapeutically effective quantity of at least one of the peptides according to one of the claims 18 to 23 is administered.

40. Electronic storage medium which contains the amino acid sequence of at least one of the peptides according to one of the claims 18 to 23 and/or the nucleic acid sequence of the nucleic acid molecule according to claim 33.

4 pages of drawings follow